University of Alberta

INTERACTIONS BETWEEN *CAMPYLOBACTER JEJUNI* AND THE HOST

by

CHRISTINE MARY SZYMANSKI

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY

EDMONTON, ALBERTA

SPRING, 1997

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced with the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-21644-6

Canada

University of Alberta

Library Release Form

Name of Author:	Christine Mary Szymanski
Title of Thesis:	Interactions Between *Campylobacter jejuni* and the Host
Degree:	Doctor of Philosophy
Year this Degree Granted:	1997

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly, or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

C Szymanski

1444 Pritchard Avenue

Winnipeg, Manitoba

Canada, R2X 0H5

January 27, 1997

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled INTERACTIONS BETWEEN *CAMPYLOBACTER JEJUNI* AND THE HOST submitted by Christine Mary Szymanski in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. G. D. Armstrong (Supervisor)

Dr. M. Belosevic

Dr. A. M. Joffe

Dr. D. Y. Kunimoto

Dr. W. M. Johnson (External examiner)

Dr. J. A. Robertson (Chair of examination)

January 20, 1997

ABSTRACT

Campylobacters are a common cause of diarrhea worldwide. *Campylobacter jejuni* accounts for approximately 95% of *Campylobacter* infections. In addition to causing diarrhea, *C. jejuni* has also been reported to cause extra-intestinal complications such as Guillain-Barré syndrome (GBS), reactive arthritis, Reiter's syndrome, meningitis, abortions, cholecystitis, bacteremia and urinary tract infections. Although *C. jejuni* is recognized as an important pathogen, very little is known about how the organism causes disease. In this study I investigated several mechanisms by which *C. jejuni* may cause disease. I found that the swimming behavior of *C. jejuni* in a viscous environment may be an important factor in the interaction of these organisms with host epithelial cells. The pH, which affects *C. jejuni* motility, may also influence the tropism of these organisms. Motility plays several key roles in *C. jejuni* pathogenesis, including increasing the efficiency of *C. jejuni* attachment to host epithelial cells. Oligosaccharide sequences probably play a subordinate role in *C. jejuni* attachment to eukaryotic cells. However, *C. jejuni* binds to lipids and may interact with lipids in host cell membranes or in the intestinal mucosa. However, lipids only partially inhibited *C. jejuni* binding to Chinese hamster ovary cells suggesting that multiple interactions occur between the bacteria and host cells. *C. jejuni* membrane proteins of 14 and 55 kDa bound to phosphatidylethanolamine. Experiments also demonstrated lipid hydrolysing activity: both phospholipase C and lipase activities in *C. jejuni* membrane preparations and acyl hydrolase and phospholipase C activities in *C. jejuni* cell extracts (cytoplasm and/or periplasm). Another *C. jejuni* 14 kDa protein demonstrated 62.8% sequence homology to the lipase chaperone proteins of *Pseudomonas* species. Antibodies against two known *Pseudomonas* lipase chaperones cross-reacted with the *C. jejuni* 14 kDa protein. *C. jejuni* possesses at least two 14 kDa proteins one of which binds phosphatidylethanolamine resulting in lipid hydrolysis or transport, and the other which may function as a lipase chaperone. All GBS patient sera

examined contained antibodies to a *C. jejuni* 14 kDa protein. Also, the fatty acid binding protein, myelin P2, may share some homology with a *C. jejuni* 14 kDa protein. Further studies are necessary to determine whether *C. jejuni* expresses a fatty acid binding protein analog and whether the *C. jejuni* 14 kDa protein plays a role in the induction of GBS. *C. jejuni* invades Caco-2 cells through a microfilament and microtubule dependent mechanism. Further studies are necessary in order to determine if calcium signalling is involved in pathogenesis.

ACKNOWLEDGEMENTS

I would like to thank the following people: my supervisor, Dr. Glen Armstrong, for his scientific input, guidance and support and my fellow group members, George Mulvey and Martin Haardt in the preparation of this thesis.

I would also like to thank San Vinh for the photography shown in this thesis as well as all the photography done throughout my program; Jack Moore for N-terminal sequencing; Dr. W. A. Boyd for his technical assistance in the videotaping experiments described in chapter 2; Dr. M. Houweling for his helpful discussions and for providing us with several of the reagents used in the study described in chapter 3; Dr. Karl-Erich Jaeger for providing LipH antibodies and protein, Dr. Steen T. Jørgensen for providing LimA antibodies and *E. coli* JA221, and Dr. Yasuhiro Yamada for providing LimL antibodies and protein described in chapter 4; and again Dr. Jaeger for the critical reading of the manuscript presented in chapter 4 and for his helpful discussions.

More generally, I would like to thank everyone else in the department of Medical Microbiology and Immunology for making my time at the University of Alberta an enjoyable one.

I would also like to acknowledge my family members Janina, Stefan and Donna and also Todd who have encouraged me along the way.

TABLE OF CONTENTS

CHAPTER 1 PAGE

INTRODUCTION TO *CAMPYLOBACTER JEJUNI*

1.1. GENERAL CHARACTERISTICS

1.1.1.	MORPHOLOGY AND GROWTH	1
1.1.2.	HUMAN DISEASE	1
1.1.3.	EPIDEMIOLOGY	2
1.1.4.	CAUSATIVE AGENTS AND INFECTIOUS DOSE	4
1.1.5.	*CAMPYLOBACTER* SURVIVAL	5
1.1.6.	HOST IMMUNE RESPONSE	6
1.1.7.	RESISTANCE TO ANTIMICROBIALS	7
1.1.8.	VACCINE DEVELOPMENT	9
1.1.9.	GENETICS	10
1.1.10.	ANIMAL MODELS	11
1.1.11.	TYPING	13

1.2. VIRULENCE FACTORS

1.2.1.	ADHERENCE	15
1.2.2.	PROTEINS INVOLVED IN ADHERENCE (PEBS)	16
1.2.3.	INVASION	17
1.2.4.	INTRODUCTION TO FLAGELLA	20
1.2.5.	FLAGELLA IN ADHERENCE AND INVASION	22
1.2.6.	FLAGELLAR ACCESSORY PROTEINS	23
1.2.7.	PILI	25
1.2.8.	CONTROVERSIAL TOXINS	26
1.2.9.	CYTOLETHAL DISTENDING TOXIN (CLDT)	28
1.2.10.	SECONDARY MESSENGER INDUCTION	28
1.2.11.	LIPOPOLYSACCHARIDE	29

1.2.12. POTENTIAL VIRULENCE PROTEINS 31

1.3. HYPOTHESIS AND OBJECTIVES 35

1.4. REFERENCES 38

CHAPTER 2

2.1. *CAMPYLOBACTER JEJUNI* MOTILITY AND INVASION OF CACO-2 CELLS

2.1.1. INTRODUCTION 59

2.1.2. MATERIALS AND METHODS 60

2.1.3. RESULTS 63

2.1.4. DISCUSSION 72

2.2. RELATED WORK 76

2.3. REFERENCES 84

CHAPTER 3

3.1. INTERACTIONS BETWEEN *CAMPYLOBACTER JEJUNI* AND LIPIDS

3.1.1. INTRODUCTION 89

3.1.2. MATERIALS AND METHODS 90

3.1.3. RESULTS 93

3.1.4. DISCUSSION 105

3.2. RELATED WORK 110

3.3. REFERENCES 114

CHAPTER 4

4.1. *CAMPYLOBACTER JEJUNI* PROTEINS INVOLVED IN LIPID HYDROLYSIS

4.1.1. INTRODUCTION 122

4.1.2. MATERIALS AND METHODS 124

4.1.3. RESULTS 129

4.1.4. DISCUSSION 143
4.2. RELATED WORK 147
4.3. REFERENCES 154

CHAPTER 5
GUILLAIN-BARRÉ SYNDROME AND *CAMPYLOBACTER JEJUNI* INFECTION
5.1. INTRODUCTION 160
5.2. MATERIALS AND METHODS 161
5.3. RESULTS 164
5.4. DISCUSSION 170
5.5. REFERENCES 177

CHAPTER 6
CAMPYLOBACTER JEJUNI IN VITRO BINDING AND INVASION
6.1. INTRODUCTION 185
6.2. RESULTS AND DISCUSSION
6.2.1. CONTROL EXPERIMENTS 185
6.2.2. SIGNAL TRANSDUCTION EXPERIMENTS 188
6.3. REFERENCES 202

CHAPTER 7
GENERAL CONCLUSIONS AND FUTURE DIRECTIONS 204

LIST OF TABLES

TABLE		PAGE
1.1.	Proteins potentially involved in virulence	34
1.2.	Summary of organisms used in studies	37
2.1.	Summary of the effect of viscosity on *C. jejuni* binding and invasion	81
3.1.	Comparison of lipid concentrations required to saturate binding of *C. jejuni* UA580 and *H. pylori* UA763	101
3.2.	Determination of *C. jejuni* UA580, *C. jejuni* ER1109, and *S. cholerasuis* SL2824 hydrophobicity	111
4.1.	Molecular weight and amino acid comparisons of *C. jejuni* UA580 identified proteins	135
4.2.	Determination of lipid hydrolysing activities in *C. jejuni* UA580 membrane preparations and cell sonicates	142
4.3.	Identification and comparison of trigger factor N-terminal amino acid sequences	151
4.4.	Summary of proteins / activities identified	153
6.1.	Comparison of cytoskeletal inhibitor experiments	194
6.2.	Summary of reagents used and observed effects on *C. jejuni* binding	201

LIST OF FIGURES

FIGURE		PAGE
2.1.	*Campylobacter* motility on swarm plates at various pH values	64
2.2.	Electron micrographs illustrating the morphology of overnight cultures of *C. jejuni* UA580 grown at (A) pH 5.0 and (B) pH 7.3	66
2.3.	Concentration-dependent increase in *C. jejuni* invasion of Caco-2 cells in CMC-supplemented MEM solutions	68
2.4.	Comparison between binding and invasion in 141 cP (0.6% w/v) CMC solutions	69
2.5.	Movements of *C. jejuni* (1-3) and *S. enteriditis* (4) in (A) regular medium and (B) viscous medium (0.6% CMC, w/v)	70
2.6.	in regular tissue culture medium (MEM) and viscous medium (0.6% CMC, w/v)	71
2.7.	Viscosity of mucus versus strain rate	77
2.8.	Actual tracing of *C. jejuni* UA580 in viscous solution	80
3.1.	Schematic diagram of carbohydrate structures associated with parental CHO cells and CHO cell mutants adapted from P. Stanley	95
3.2.	Comparison of *C. jejuni* binding and invasion of CHO cells and CHO cell mutants	96
3.3.	*C. jejuni* binding to CHO cells in the presence of simple carbohydrates	97
3.4.	Analysis of lipids extracted from CHO cell membranes	100
3.5.	Affect of varying concentrations of tetramethylurea on the binding of *C. jejuni* to ELISA wells coated with 100 ng PC	102
3.6.	Schematic diagram illustrating the relative affinities of *C. jejuni* for lipids and lipid derivatives	103
3.7.	Modified TLC overlay	104

3.8.	*C. jejuni* binding to CHO cells in the presence of PC/oleate vesicles	106
3.9.	Binding of *C. jejuni* UA580 specifically to PC	113
4.1.	SDS-PAGE of crosslinking experiment between ^{125}I-SASD-derivatized phosphatidylethanolamine (PE) and *C. jejuni* UA580 whole cells (WC) and membrane preparations (MEMB) with (+) or without (-) excess underivatized PE	131
4.2.	^{3}H-PC autoradiogram demonstrating the presence of lipid binding activity in *C. jejuni* UA580 membrane preparations	134
4.3.	Lipase chaperone crossreactivity experiment	137
4.4.	Native egg-yolk overlay gel with *C. jejuni* UA580, *C. jejuni* 81116, *C. jejuni* E863, and *C. jejuni* ER1109 whole cell lysates	139
4.5.	Determination of lipid hydrolysing activity in *C. jejuni* UA580 whole cell lysates (WCL), supernatants (SUP), total membranes (MEMB), inner membranes (IM), and outer membranes (OM) by egg-yolk overlay in native gels	140
4.6.	Determination of the sensitivity of *C. jejuni* UA580 lipid hydrolysing activity to temperature, protein digestion, and disulfide-bond reduction by egg-yolk overlay in native gels	141
4.7.	Egg-yolk overlay of a *C. jejuni* UA580 membrane preparation separated by gel electrophoresis in the presence of SDS	148
4.8.	Preparation for N-terminal sequencing	149
5.1.	Comparison of antibody crossreactivity with *C. jejuni* whole cell lysates	165
5.2.	Silver stained polyacrylamide gel demonstrating the binding of a14 kDa *C. jejuni* membrane protein to PC	166
5.3.	Human antibody reactivities to *C. jejuni* whole cell lysates	168
5.4.	Human antibody reactivities to the adipocyte lipid binding protein	169
5.5.	TLC overlay comparing antibody responses to various lipids	171

6.1.	Composite demonstrating *C. jejuni* UA580 invasion of Caco-2 cells	187
6.2.	Effect of Triton X-100 on the viability of *C. jejuni* E863	189
6.3.	*C. jejuni* UA580 invasion timecourse in Caco-2 cells	190
6.4.	*C. jejuni* E863 invasion of Caco-2 cells in the presence of cytoskeletal inhibitors	192
6.5.	Binding and invasion of *C. jejuni* E863 to Caco-2 cells in the presence of cytoskeletal inhibitors	193
6.6.	*C. jejuni* E863 Caco-2 cell invasion in the presence of verapamil	197
6.7.	*C. jejuni* E863 binding and invasion of Caco-2 Cells in the presence of 100 μM verapamil	198
6.8.	*C. jejuni* UA580 binding to verapamil-coated wells	200

LIST OF ABBREVIATIONS

14:0	myrystic acid
16:0	palmitic acid
18:0	stearic acid
18:1	oleic acid
20:4	arachidonic acid
ALBP	adipocyte lipid binding protein
Asn	asparagine
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHI	brain heart infusion
BSA	bovine serum albumin
Ca	cardiolipin
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CFB	cell binding fraction
CFU	colony forming units
Ch	cholesterol
CHO	Chinese hamster ovary
Cj	*C. jejuni*
CLDT	cytolethal distending toxin
CMC	carboxymethylcellulose
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
CT	cholera toxin
CTP	cytidine 5'-triphosphate

DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAE	experimental allergic encephalomyelitis
EAN	experimental allergic neuritis
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethylether) N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
FABP	fatty acid binding protein
FBS	fetal bovine serum
FDA	food and drug administration
fla+	flagellated
fla-	unflagellated
Fur	ferric uptake regulatory
Ga	gangliosides
GBS	Guillain-Barré syndrome
GlcNAc	N-acetylglucosamine
GPC	deacylated PC
GPC	glycerylphosphorylcholine
GTP	guanosine 5'-triphosphate
H-7	1-(5-isoquinolinylsulfonyl)-2-methylpiperazine
HL	heat-labile
ILBP	ileal lipid binding protein
IODO-GEN	α-1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril-coated

L-FABP	liver fatty acid binding protein
LBP	lipid binding protein
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LT	labile toxin
LysoPC	lysophosphatidylcholine or monoacyl-PC
LysoPE	lysophosphatidylethanolamine
MAG	monooleolylglycerol
Man	mannose
MapA	membrane associated protein
MEE	multilocus enzyme electrophoretic typing
MEM	minimal essential medium
MHC	major histocompatibility complex
MOMP	major outer membrane protein
NBT	nitro blue tetrazolium
nsL-TP	non-specific lipid transfer protein
PA	phosphatidic acid
PAL	peptidoglycan-associated lipoprotein
PBS	phosphate buffered saline
PC	phosphatidylcholine
PC-TP	phosphatidylcholine transfer protein
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEB	Pei, Ellison and Blaser
PflA	paralysed flagella
PG	phosphatidylglycerol
PGE_2	prostaglandin E2

PI	phosphatidylinositol
PI-TP	phosphatidylinositol transfer protein
PKC	protein kinase C
PLA_1	phospholipase A_1
PLA_2	phospholipase A_2
PLB	phospholipase B
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
PS	phosphatidylserine
REA	restriction endonuclease analysis
RILT	rabbit ileal loop
RITARD	removable intestinal tie adult rabbit diarrhea
SASD	Sulfosuccinimidyl 2-(*p*-azidosalicylamido) ethyl-1-3-dithiopropionate
SCP2	sterol carrier protein 2
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sIgA	secretory IgA
SE	standard error of the mean
SOD	superoxide dismutase
SP	sphingomyelin
TAG	triacylglycerol
TLC	thin layer chromatography
TMU	tetramethylurea
TTP	thymidine 5'-triphosphate
Tween 20	polyethylenesorbitan monolaurate 20

USA	United States of America
v/v	volume per volume
VT	verotoxin
w/o	without
w/v	weight per volume
WT	wild-type

CHAPTER 1

INTRODUCTION TO *CAMPYLOBACTER JEJUNI*

1.1. GENERAL CHARACTERISTICS

1.1.1. MORPHOLOGY AND GROWTH

Campylobacters were initially classified in the family *Vibrionaceae* but now comprise a new genus known as *Campylobacter* in the family *Spirillaceae* (126). Although there are several species in the *Campylobacter* genus, *Campylobacter jejuni* is responsible for 95-98% of the reported cases of *Campylobacter*-induced gastroenteritis (119). The organisms are curved or spiral rods ranging from 1.4-3.5 μm in length and 0.2-0.6 μm in width (86, 126). Most organisms have bipolar unsheathed flagella ranging from 2.6-3.9 μm in length and approximately 21 nm in width and exhibit corkscrew-like motility (86). *Campylobacter* morphology is rather pleomorphic. In a report by Griffiths, exponentially growing cells exhibited typical short spiral morphology. However, cells at mid-stationary phase were approximately twice the length of exponential cells. In the late-stationary / early death phase two morphologies were observed: coccal forms and cells 3-4 times the length of exponential forms. Upon continued incubation predominantly coccal forms were present (38). Campylobacters require microaerophilic (5% O_2 optimal) and capnophilic (3-5% CO_2) conditions for growth (33). *Campylobacter jejuni* grows best at 42°C but will also grow at 37°C. The organism grows slowly with reported doubling times from 1 hour to 6 hours (27, 54).

1.1.2. HUMAN DISEASE

The most common clinical symptom of *C. jejuni* infection is diarrhea but other symptoms such as fever and chills, abdominal pain, nausea, vomiting, weight loss, and malaise may also occur (68, 69). The infection results in acute inflammatory enteritis affecting both the small intestine (jejunum and ileum) and the colon (69). *Campylobacter*

enterocolitis is characterized by inflammatory cells, villus degeneration and atrophy, loss of mucus, crypt abscess, and ulceration of mucosal epithelium (28). The disease ranges from watery diarrhea to severe dysentery-like illness (bloody stools with mucus and fecal leukocytes) associated with an intestinal inflammatory response (12, 21). However, the cause of the mucosal damage and inflammatory response remains unclear. The incubation period ranges from 1-7 days and diarrhea, which is usually self-limiting, lasts from 2-7 days. However, the symptoms may persist for 1-3 weeks in up to 20% of patients (69) and may also persist longer in the case of immunocompromised patients (32). Convalescent fecal excretion of organisms can last for 2 weeks to 3 months (126). Death due to infection is rare (69).

Although *C. jejuni* infection primarily results in self-limiting diarrhea, there have been reports of extra-intestinal complications such as Reiter's syndrome, reactive arthritis, Guillain-Barré syndrome (GBS), meningitis, abortions, cholecystitis, transient bacteremia and urinary tract infections (33). Approximately 20-40% of patients with GBS are infected with *C. jejuni* 1-3 weeks prior to neurological symptoms (69). However, there is no relation between the severity of symptoms and development of GBS (69). Bacteremia is noted in less than 1% of cases (16). However, *Campylobacter* bacteremia may be life-threatening in malnourished children. Also, a recent history of diarrhea is not a prerequisite for the development of *Campylobacter* bacteremia since the organisms are known to be present in the gastrointestinal tract of asymptomatic children in developing countries where such infections are endemic (99).

1.1.3. EPIDEMIOLOGY

When *Campylobacter* was first isolated from diarrheal stools from humans in 1972, it became evident that Campylobacters are a common cause of diarrhea worldwide (69). *Campylobacter* is the leading cause of acute gastroenteritis in humans around the world with an estimated 400 million cases annually (40). *C. jejuni* is the most frequently

encountered species in human enteritis. In the USA, illnesses due to *Campylobacter* are more common than illnesses due to *Salmonella* spp. and *Shigella* spp. combined (33). In the USA, annual cases are estimated to be in excess of 2 million (81). Also, the incidence of *C. jejuni* infection in AIDS patients exceeds that of the normal population several fold (32). In developed countries incidence peaks in infancy and in early adulthood with a slight predominance in males (69, 113). Most cases occur sporadically in the USA but outbreaks do occur (73). Rates of *C. jejuni* infection peak in summer and early fall but cases occur throughout the year (69). The reason for seasonal patterns is unknown. However, the carriage rates in chickens is higher in the summer than in the winter and activities such as barbeques and drinking untreated water while hiking and camping may also be risk factors (113). Deaths attributable to *C. jejuni* in the USA are estimated to be from 120 to 360 per year (113) to 2.4 per 1000 reported cases (69).

Campylobacter infections are hyperendemic among children in developing countries (69) and diarrheal diseases are among the leading causes of childhood morbidity and mortality in these areas of the world (62). Diarrheal diseases rank third after malaria and measles as the most important causes of morbidity in children below the age of 5 (62). Annually, at least 750 million diarrheal cases with a calculated mortality rate of 4-5 million children occur in these countries (62). It is estimated that children spend 15-20% of their first 2 years with a diarrheal illness (62). After enterotoxigenic *E. coli* and rotavirus, *C. jejuni* ranks as the third most common cause of diarrhea in children in developing countries (112). Two differences have been observed in the epidemiology of *C. jejuni* infection in developed and developing countries. *C. jejuni* gastroenteritis is reported in all ages in developed countries while it is most prevalent in infants and children of developing countries (68). Also, in developing countries, *C. jejuni* is associated with acute secretory diarrhea while in developed countries the infection is mostly inflammatory in nature (9).

Campylobacter is also a significant cause of traveler's diarrhea in the USA with annual infection rates from 5-10% (113) to 25-37% (in highly endemic areas) (40) in

comparison to about 50% in Scandinavia. *Campylobacter* is second only to enterotoxigenic *Escherichia coli* (ETEC) as a cause of traveller's diarrhea (100). Outbreaks and sporadic cases are also well documented among the USA military personnel (40).

1.1.4. CAUSATIVE AGENTS AND INFECTIVE DOSE

Campylobacter enteritis is a zoonotic disease (115). The organisms occur usually as commensals in cattle, pigs, sheep, poultry, pets, birds, apes, rodents and even insects (116). Shellfish such as clams may also become contaminated by feeding in waters polluted by farm runoff (27). Person-to-person transmission appears to be uncommon but reports of nosocomial spread, intrafamilial clusters, and infections in neonates whose mothers had positive cultures suggest that it may occur (49). Exposure to diarrheic pets or other infected animals is associated with an increased risk of *C. jejuni* enteritis (107, 113).

There appear to be several pathways of infection. *Campylobacter* can survive in the environment for several weeks and cause infection when contaminated untreated water or milk are consumed (113). Reports show a high incidence of *C. jejuni* in the intestines of poultry, cattle and pigs at slaughter (33). The principal vehicle of transmission is raw or undercooked meat (113). Studies have shown that more than 70% of sporadic infections are associated with eating chicken thus making chicken the single most important vehicle of transmission in developed countries (69, 73). Outbreaks are most frequently due to consumption of raw milk or improperly treated water (69, 81).

When *Campylobacter* carriage in chicken flocks is determined, the organism is usually isolated in large numbers from the majority of birds sampled (115). Retailed chickens display contamination rates between 60-80%, with *Campylobacter* counts in the range of 10^6 per fresh chicken carcass (100 fold lower in frozen birds) (113). Although *Campylobacter* is excreted in chicken feces, the organism is not vertically transmitted to the egg (115). Chickens, in a commercial production, remain free of *Campylobacter* for several weeks but eventually all become colonized in the cecum (115). Attempts are

currently being made to reduce *Campylobacter* colonization in chicks by oral administration of defined competitive exclusion cultures (a combination of *Citrobacter diversus*, *Klebsiella pneumoniae*, and *Escherichia coli*) (109).

Studies in human volunteers suggest that between 800 and 10^6 organisms may produce symptoms in 10-50% of individuals (11, 69). However other reports suggest that as few as 500 bacteria can cause infection (21, 33).

1.1.5. *CAMPYLOBACTER* SURVIVAL

Campylobacter jejuni is a fastidious organism that encounters harsh environmental conditions and host defense mechanisms. Yet, the organism survives in several milieus and is very successful in causing infection. This section describes what is known about the survival of *Campylobacter* in potential biological reservoirs.

C. jejuni is not able to survive in acidic solutions with a pH less than 3 however, solutions with a pH greater than 3.6 had no effect on *C. jejuni* survival (13, 126). The organism was also shown to multiply and survive in bile for 2 months at 37°C (13). *Campylobacter* survives in urine for less than 48 hours at 37°C but was shown to survive in urine for several weeks at 4°C (13). *Campylobacter* survival is also better in feces, milk, and water kept at 4°C rather than at 25°C (13, 126). It was shown that *C. jejuni* with intact DNA can persist for extended periods of time in poultry water supply systems (81). Interestingly, one report described *C. jejuni* adaptation to an aerobic metabolism but this observation has not been confirmed (48). Although *C. jejuni* has been isolated from the intestines of poultry, cattle and pigs at slaughter, poultry is the major cause of infection (see above). It is believed that *C. jejuni* is eliminated through drying when pork and beef are processed (16). The high incidence of poultry carcass contamination by *Campylobacter* may be due to the different method of processing (16). Irradiation is an effective method for killing *C. jejuni* on poultry carcasses and the necessary levels of radiation have been

sanctioned by the USA FDA. However this method is not presently in use because of consumer concerns over the safety of irradiated poultry (27).

In summary, *C. jejuni* is sensitive to drying, storage at 25°C, acidic conditions below pH 3.0, aeration, and heat. *C. jejuni* also cannot propagate at temperatures below 30°C so the organism is unlikely to grow on food at room temperature (16, 23). However, survival in solutions with a pH of >3.6, cool water and refrigerated foods (such as milk and meat products) will promote infections with *Campylobacter jejuni*.

1.1.6. HOST IMMUNE RESPONSE

Serum antibodies against *C. jejuni* are first detected 6-7 days after onset of illness-approximately 10 days after initial infection (77). IgA levels peak about 7-10 days before the IgG peak at about 3-4 weeks (77). The IgM levels peak earlier than IgG (77). After the initial response, both IgA and IgM antibodies decline rapidly while IgG antibodies persist for weeks (77). The intestinal mucosa initiates a rapid local antibody response (77). The importance of secretory IgA (sIgA) at the mucosal surface has been demonstrated in studies where sIgA antibodies inhibited bacterial adherence to epithelial cell monolayers (65). Gastrointestinal peristalsis is also believed to be an important nonspecific host defense, especially in the clearance of sIgA agglutinated bacteria (21). The bactericidal activity of human serum is mediated by both antibody and complement (21). The effective complement dependent bactericidal activity in normal human serum is probably due to several antigenic determinants such as flagella, LPS, and the heat shock proteins, GroEL and GroES, which are shared between *C. jejuni* and other bacterial species (43, 83, 135). Serum sensitivity may play an important role in preventing bacteremia and extraintestinal infections (77).

There is a considerable amount of evidence suggesting that the humoral antibody response is enough to provide protective immunity. In developing countries, asymptomatic infection is common in as many as 40% of children and isolation rates are much lower in

older persons (21). The decrease in the illness-to-infection ratio with age in developing countries is paralleled with the increasing levels of *C. jejuni* antibodies in serum (77). When adults in developing countries are symptomatic, the illness tends to be milder without inflammatory diarrhea. This observation suggests that *Campylobacter* infection is intense in infancy with a rapid development of immunity (21). Again, in developing countries, maternal antibodies have been demonstrated to provide protection against *Campylobacter* spp. since *Campylobacter* has been isolated from non-diarrheic stools of neonates (122). Breast fed infants are more protected against *Campylobacter* infection but high proportions of infants develop symptomatic infections once weaned. In developing countries such as Thailand, infection is highest in children <1 yr of age and the case-to-infection ratio falls with age (120). In general in developing countries, *Campylobacter* immunity results in mostly asymptomatic infections beyond the age of 3 (69).

In volunteer studies in the USA, ill volunteers developed serum antibody responses and were protected from illness but not reinfection with the same strain (11). However, the widespread incidence of asymptomatic infection in developing countries suggests that persistent exposure to multiple strains allows development of protective immune responses against common epitopes (77). Also, diarrhea due to *Campylobacter* is much lower in chronic drinkers of raw milk in the USA suggesting immunity may be induced by recurrent exposure (88). However, epidemiological data suggest that humans become resistant only to clinical disease but not to intestinal colonization (10).

1.1.7. RESISTANCE TO ANTIMICROBIALS

Resistance to kanamycin, chloramphenicol and tetracycline is plasmid mediated and the resistance genes have been cloned and sequenced (118, 121). Resistance to erythromycin is considered to be chromosomally mediated (117). Resistance in *C. jejuni* is frequently mediated by self-transmissible plasmids. Fortunately, the host range of the plasmids is narrow since they can transfer to and be maintained in only closely related

Campylobacter species (117). For example, the plasmid mediated resistance to chloramphenicol is very rare among *Campylobacters* (117).

Different patterns of *Campylobacter* antibiotic resistance are being reported around the world. In Thailand, approximately 53% of Campylobacters are resistant to erythromycin however it has been reported that only 2% are resistant to erythromycin in developed countries (27). Erythromycin resistance rates are approximately 10% in Sweden (133). Importantly, the development of resistance to macrolide antibiotics *in vivo* was observed in an AIDS patient treated with clarithromycin (32). The authors show that the infection occurred with a susceptible strain which later became resistant rather than by two strains with different resistances (32). In contrast, a report from Spain showed that macrolide activity against *Campylobacter* spp. is stable but resistance to quinolones has rapidly developed over the last 5 years (108).

Tetracycline resistance in Japan has reached 55% (133) whereas in other developed countries up to 25% of strains are resistant to this antimicrobial (69). Reports also demonstrate an 8% resistance rate to nalidixic acid in Bangladesh and the emergence of fluoroquinolone resistance in Denmark (133). The isolation of a strain with multiple antibiotic resistances was reported. This isolate was susceptible to aminoglycosides (gentamicin and neomycin) and to chloramphenicol but resistant to nalidixic acid and fluoroquinolones (ciprofloxacin and ofloxacin), tetracycline, ampicillin, trimethoprim, and macrolides (erythromycin, clarithromycin, azithromycin) (133). It is not surprising that increases in resistance rates have been shown to correspond to increases in antibiotic administration.

Since most *Campylobacter* infections are self-limiting, antibiotic treatment should only be administered to patients with prolonged or worsening symptoms, in pregnancy, and to immunosuppressed individuals (69, 133). In the USA, erythromycin is the treatment of choice (69).

1.1.8. VACCINE DEVELOPMENT

Since *Campylobacter* infection is generally self-limiting, the need for the development of a vaccine may not be obvious. However, cost estimates described at a recent GBS conference suggested that *Campylobacter* vaccination may become important in the future. Also, *Campylobacter* infection was reported to be the main cause of diarrhea in U. S. troops deployed to Thailand (72). Therefore, vaccination of certain specialty groups such as the U.S. military and also travellers may be needed.

Little is known about which *Campylobacter* antigens are protective so the first vaccine to be tested in human volunteers consisted of killed whole bacterial cells mixed with adjuvant. The adjuvant chosen was ETEC heat labile toxin which, when administered orally with sonicated whole cell antigens, elicits a protective immune response against live organisms (100, 127).

However, emerging evidence suggesting that *C. jejuni* cross-reactive epitopes react with human neuronal antigens necessitates the development of an acellular vaccine (see Chapter 5). When protective antigens are identified, it will be possible to develop a second generation of subunit vaccines. Potential vaccine candidates include flagellin, major outer membrane protein porin (MOMP), and adhesive proteins known as PEBs (see 1.2.2). Flagella are important for motility and cellular invasion. Flagella are antigenic and immunodominant and antibodies against flagella have been shown to be partly protective (77). However, flagella also exhibit phase variation, antigenic variation, and posttranslational modifications (see 1.2.4 and 1.2.6). The MOMP is also immunogenic with possible conserved epitopes and antibodies against the porin may interfere with several metobolic processes (77). The 28-31 kDa adhesive proteins known as PEBs have also been shown to be antigenic (77). However, more work needs to be done in identifying potential vaccine candidates.

Recently a *C. jejuni recA* mutant was described for inclusion into attenuated vaccines (40). In *V. cholerae*, *recA* is responsible for enhancing the virulence of the

organism by amplifying the cholera toxin genes. Also, *Salmonella typhimurium recA* mutants have been shown to be avirulent. In this study the *C. jejuni recA* mutant was still capable of colonizing and eliciting protection in a rabbit model (40). Interestingly, the mutation caused the loss of detectable natural transformation which would be beneficial in the design of an attenuated vaccine (40). Defects in natural transformation would prevent organisms from acquiring and incorporating virulence or resistance genes from the environment. Vaccine development is in progress.

1.1.9. GENETICS

The genome of *C. jejuni* consists of a single circular DNA molecule which is approximately 1.7 Mb in size (18, 118). *Myxococcus xanthus* has the largest genome at 9.45 Mb while *Mycoplasma genitalium* has the smallest genome at 585 kb (118). The *C. jejuni* genome is 36% of the size of the *E. coli* chromosome (123). Plasmids of varying sizes have been detected in *C. jejuni* but have only been shown to contain antibiotic resistance (21, 126). The G+C content of *C. jejuni* is 32 mol% (118).

Methods of introducing new DNA into *C. jejuni* include the use of vectors: transfer of shuttle vectors from *E. coli* to *C. jejuni* by conjugation, use of suicide vectors resulting in homologous recombination and *Campylobacter* plasmids; genetic exchange through conjugative plasmids and natural transformation; electrotransformation (electroporation) using high voltages to induce DNA uptake; and bacteriophage transduction (118, 128, 130). Most methods of introducing foreign DNA into *Campylobacter* spp. are successful with a limited number of strains. Sometimes, however, transfer occurs at low frequency (131). Natural transformation of bacterial chromosomal DNA is the most efficient method for gene manipulation in competent strains of *C. jejuni* such as the widely used *C. jejuni* 81116 (131).

Natural transformation involves bacterial uptake of DNA from the environment and incorporation of the DNA into its genome (128). Gram negative organisms take up only a

few molecules of homologous DNA while heterogeneous DNA is taken up at a lower frequency (128). Gram positive organisms bind and take up a large number of DNA molecules regardless of the source (128). However, natural transformation of plasmid DNA is normally rare in both bacterial types (128). *Campylobacters* are unique among enteric pathogens because they are naturally transformable (40). Wang showed that most *C. jejuni* strains are naturally competent for transformation during the log phase of growth (128). The organisms show a strong selectivity for the uptake of their own DNA (128). The frequency of plasmid DNA uptake is approximately 1000 times lower than the uptake of chromosomal DNA however, if the recipient contains a homologous plasmid, transfer rates increase (128). Other organisms possess DNA uptake sequences which are necessary for binding and uptake into cells but these sequences have yet to be identified for *Campylobacter* (118).

1.1.10.ANIMAL MODELS

An animal model for *C. jejuni* infection has been difficult to find. Most models did not reflect the full spectum of human disease (mice and hamsters) or, where successful infections were observed, resulted in the use of time consuming techniques (removable intestinal tie adult rabbit diarrhea [RITARD] model) or expensive animals (non human primates). Several *in vivo* models of *Campylobacter* infection have been described including gnotobiotic beagle puppies, young rhesus monkeys, RITARD model, Syrian hamsters, mink, ferrets, mice, newborn piglets, and chicks.

When adult Syrian hamsters were challenged with *C. jejuni*, none of the animals developed illness (1). However, the authors observed a significant difference in the ability of wild-type *C. jejuni* to colonize hamsters compared with the non-motile non-flagellated *C. jejuni* mutants (1). Also, some animals that were challenged with the non-motile non-flagellated mutant excreted only motile, flagellated wild-type Campylobacters in their feces

(1). Laboratory mice, like hamsters, also show signs of colonization and sometimes bacteremia but diarrhea is not observed (31).

A key factor in the RITARD model is that colonization of the mucosa is enhanced through the temporary inhibition of normal peristaltic clearance mechanisms (127). Again there is no sign of disease but bacteremia occasionally develops. However the rabbit naturally clears the organism and develops an immune response. It was demonstrated that rabbits fed as few as 100 organisms became intestinally colonized and resistant to recolonization with the homologous but not heterologous strain. The RITARD model has been useful but requires surgical intervention and is time consuming (31).

Even with high *Campylobacter* colonization rates in poultry, infected animals show little or no clinical signs of illness (60). However, experimental infection of newly hatched chickens with *C. jejuni* isolates from chickens or turkeys produced illness and mortality (60). In another study diarrhea, weight loss and mortality was observed after infecting 3-day old chicks (102). The minimun infective dose for causing diarrhea in 90% of the chickens was as few as ninety bacteria. Also, electron microscopy and immunofluorescence microscopy showed *Campylobacter* inside epithelial cells (see 1.2.3).

Infected newborn piglets and gnotobiotic puppies showed signs of diarrhea and physiological symptoms similar to that observed in human infection (8, 31). In the newborn piglet model, electron microscopy demonstrated the presence of intracellular bacteria (8). Mink have also been shown to develop severe *C. jejuni* colitis (31).

The most successful infections were observed with non human primates. Non human primates display many of the clinical and epidemiological features of human infections, especially cases described in developing countries (31). For example, experimental challenge of pig-tailed macaques with a virulent strain of *C. jejuni* produced an acute self-limiting diarrhea (103). Intestinal biopsies showed similar colon lesions as those reported from humans. Luminal epithelium exhibited degeneration with replacement of columnar epithelium with flattened cells and loss of goblet cells. Although intestinal

colonization was not inhibited by previous infection, the animals did not exhibit clinical illness. High titers of *Campylobacter* were present in stools of challenged animals but the duration of excretion was less. Passive protection was also evident in this model since none of 23 neonates nursed by mothers developed diarrhea.

Experimental infection in monkeys was characterized by secretory diarrhea, bloody stools, and fecal leukocytes lasting 7-11 days (105). *C. jejuni* was excreted 2-4 weeks postchallenge. Mild diarrhea occurred after rechallenge with the same strain or heterologous strain. Monkeys which had experienced multiple infections did not exhibit illness when challenged. Results indicated clinical and pathological similarity to human infection and that prior infection protects against subsequent challenge with the homologous strain.

Young weanling ferrets are the current model of choice being increasingly used as an *in vivo* model system for *Campylobacter* infection (31). The ferrets have mucoid, sometimes bloody or watery self-limiting diarrhea that can be consistently reproduced.

1.1.11.TYPING

Understanding the epidemiology of *Campylobacter* infection depends on methods that discriminate within species and distinguish strains from different sources (85). The most common serotyping methods involve heat-labile (HL) antigens or heat-stable (O) antigens (73). Two independent groups, Penner and Hennessy and Lauwers *et al.* developed serotyping schemes on the basis of soluble, heat-stable antigenic factors using a passive hemagglutination technique with unabsorbed antisera. The serospecific determinant of the thermostable scheme is lipopolysaccharide (LPS). For the heat-labile antigen, Lior *et al.* developed a slide agglutination scheme differentiating on the basis of heat-labile antigenic factors using live whole cells and antisera absorbed with heat-stable preparations from homologous serostrains (2).

It was previously believed the flagellar protein was an essential determinant of the heat labile antigen typing scheme since nonflagellated mutants lost the ability to be serotyped by the Lior procedure (41, 132). In order to test this hypothesis, flagellin mutants were moved from the original host to a number of other Lior serogroups by natural transformation. The results of the transformation showed that a nonflagellar antigen is often the serodeterminant in heat-labile Lior serotyping scheme (41). Although the determinant responsible for the serogroup specific epitopes has not been identified, both serotyping schemes are still the method of choice. However, the necessary antisera and reagents are not commercially available for the serotyping systems of Penner and Hennessy and Lior *et al.* which require as many as 60 and 108 antisera respectively (85). Modifications to the serotyping scheme by incorporating 24 of the most prevalent O and 23 of the most prevalent HL serotypes was proposed to be useful for outbreak management and for surveillance (84).

Other techniques developed over the past years include: biotyping, bacteriophage typing, lectin typing, plasmid analysis, auxotyping, resistotyping, outer membrane protein analysis by gel electrophoresis, multilocus enzyme electrophoretic typing (MEE), 16S and 23S rRNA probing (ribotyping), PCR fingerprinting, and bacterial restriction endonuclease analysis (REA) of chromosomal DNA (34, 73, 85).

Patton *et al.* compared several typing schemes. The advantage of using MEE is that not only can strains be identified and typed but the genetic relatedness among strains can be determined (85). The group found the HS and HL serotyping methods were as successful as the most sensitive genotypic procedures in determining the relationship between organisms within each epidemic (85). However, they proposed that genotypic methods have an advantage over phenotyping. Genotypic methods measure stable chromosomal differences so they have the potential of producing more consistent, reproducible results which can be applied to other species or organisms. The difficulty with genotypic methods is that the procedures are complex and require special equipment and reagents while

serotyping is a more rapid and simple procedure (85). In contrast, Khakhria *et al.* suggested that the number and diversity of phage types makes phage typing the method of choice in epidemiological studies (52).

Nachamkin *et al.* developed another molecular typing system for *C. jejuni* and *C. coli* based on restriction fragment length polymorphism analysis of the flagellin gene, *flaA* (73). They recently extended the typing scheme to include more flagellin types (74).

Since *C. jejuni* and *C. coli* are among the most frequently isolated Campylobacters causing diarrhea in humans and since isolation from clinical samples requires long incubation times and special growth conditions a simpler method of differentiation between the two is necessary (116). Stucki *et al.* identified a 22 kDa membrane associated protein (MapA) which is unique to *C. jejuni*. Evidence suggests that MapA is an inner membrane lipoprotein. Antiserum against MapA reacted only against *C. jejuni* but not *C. coli*.

1.2. VIRULENCE FACTORS

1.2.1. ADHERENCE

C. jejuni associates with M cells of Peyer's patches and is also observed to be closely associated with or internalized in enterocytes (30). However there is no evidence for preferred association with M cells (106). The search for the host receptor involved in mediating attachment has been unsucessful. Most inhibition experiments with simple carbohydrates as well as with certain lectins indicate that these compounds do not play a role in the attachment process (70, 76). However, these observations do not eliminate the possibility that other complex carbohydrates or lectins may play a role in attachment. The role carbohydrates play in adherence remains controversial as other groups are able to inhibit *C. jejuni* binding with simple sugars (19, 71, 104). Also, there has been one report that suggests that extracellular matrix components may serve as anchor molecules for *C.*

jejuni adhesion (59). All *C. jejuni* strains are negatively charged and expose a hydrophobic surface (125). *C. jejuni* isolates with a high negative surface charge and weak hydrophobic surface adhered better to enterocyte-like HT-29 cells than strains with less charge and a more hydrophobic surface (125). Sialylation of flagella and LPS may play a role in *C. jejuni* surface charge and adherence (see below).

1.2.2. PROTEINS INVOLVED IN ADHERENCE (PEBS)

Several *C. jejuni* structures (flagella, LPS, MOMP) were once believed to be involved in aherence (see below). These structures are now known to perform different functions unrelated to adherence. However, the best characterized proteins that may be involved in adherence are the PEBs which were named after the investigators that first identified them (Pei, Ellison and Blaser).

In 1990, deMelo and Pechère identified *C. jejuni* surface proteins which adhered to eukaryotic cells *in vitro* (24). Two of these proteins with apparent molecular masses of 28 kDa and 32kDa were later identified as PEBs. Fauchère *et al.* also identified two proteins in cell binding fractions (CFB's) with apparent molecular masses of 27 kDa (CFB1) and 29 kDa (CFB2) (30). CFB1 and CFB2 were later shown to be PEB1 and PEB4 respectively (15).

Four proteins from *C. jejuni* 81-176 were identified by acid extraction: 28 kD (PEB1), 29kD (PEB2), 30kD (PEB3) and 31kD (PEB4) (89). All four PEB proteins were removed by gentle extraction with low pH glycine suggesting that they are not transmembrane proteins (88). PEB2 and PEB4 are not major targets for the immune response (88). Trypsin digestion did not remove PEB4 suggesting that the protein is not surface exposed (88). PEB1 and PEB3 are antigenic. PEB1 is common in all *C. jejuni* and *C. coli* cells. PEB3 exhibits sequence homology to class I pili from *Neisseria meningitidis* and *Escherichia coli* heat-labile enterotoxin B subunit both of which are

involved in binding (89). PEB1 and PEB3 are immune targets during natural infection but it is unknown whether the resulting immune response will be protective.

Immunogold labelling showed that PEB1 is surface exposed while PEB4 is not (51). PEB1 adhered more to HeLa cell membranes than did PEB4. Also, *C. jejuni* adherence to HeLa cells was reduced with PEB1 antiserum whereas PEB4 showed background levels of inhibition. The authors also demonstrated that hydrophobic interactions participate in the adherence of both proteins, especially PEB4 to HeLa membranes.

Sequence analysis of PEB1 demonstrated homology to amino acid binding proteins (87). However, PEB1 is unique since all identified binding proteins in bacterial transport systems are located in the periplasmic space while PEB1 is surface exposed. It is also interesting that the PEB1 preprotein contains both signal peptidase I and II cleavage sites (87). PEB4 copurifies and coadheres with PEB1 but is not surface-exposed and does not bind to cells. Sequence analysis of the conserved periplasmic protein suggests that the protein is possibly involved in protein transformation (possible export or protease activity) (15). PEB1 is the only PEB that has been shown to play a major role in cell adherence. Further study on the roles of the PEBs in adherence and protection are still needed.

1.2.3. INVASION

Invasion of epithelial cells is an essential virulence mechanism of several enteric bacteria such as *Salmonella* spp., *Shigella* spp., enteroinvasive *E. coli*, and *Yersinia* spp. Electron micrographs of *C. jejuni* infection in infant *Macaca mulatta* show that cell invasion is a primary mechanism of colon damage and diarrheal disease (106). Also, the primary mechanism of colitis in infant rhesus monkeys is associated with invasion into absorptive cells in the colon (104). Immunohistochemical staining with *Campylobacter* antiserum showed invasion of *Campylobacter* in human colonic mucosa (124). *C. jejuni* infection is often characterized by the presence of blood and leukocytes in stool and mucosal

ulcerations and crypt abscesses in rectal biopsy specimens (58). Several studies have confirmed the existence of invasive strains and demonstrated a strong association with illness, especially inflammatory diarrhea (101). When piglet intestinal epithelial cells, which are anatomically and physiologically similar to those in humans, were infected with *C. jejuni*, the recovered colonies invaded INT 407 cells at higher frequency than the parental strain (8). It has been suggested that invasiveness may be an *in vivo* virulence determinant.

Chloramphenicol, which is not toxic to eukaryotic cells and which does not affect bacterial motility, acts as a selective inhibitor of bacterial protein synthesis (130). In experiments with chloramphenicol, three independent groups demonstrated that *C. jejuni* invasion but not binding was significantly reduced (53, 80, 130). These observations suggest that bacterial protein synthesis is required for invasion. However, the results obtained by Russell *et al.* were clearly not consistent with those of these other groups showing that invasion was not inhibited by chloramphenicol (104). Cocultivation of *Campylobacter* with human epithelial cells resulted in enhanced invasion (53). The response was shown to be due to factors or conditions present in the culture medium and also to the direct interaction of *Campylobacter* with INT 407 cells (53). Antiserum raised against *C. jejuni* cultivated with INT 407 cells inhibited invasion but not adhesion in a dose dependent manner. These results also suggested that new proteins, synthesized during cocultivation, may play a role in invasion (56).

Reports have shown that *C. jejuni* invasion of eukaryotic cells is accompanied by a dense accumulation of microfilaments (106). Konkel *et al.* demonstrated that invasion required microfilaments since both cytochalasin B and cytochalasin D inhibited invasion (54, 55). The results they obtained for microtubule involvement were less clear. Colchicine exhibited non-concentration dependent inhibitory effects on invasion (54). Oelschlaeger *et al.* demonstrated that microfilament *and* microtubule pathways were involved in invasion. They speculated that the pathway utilized may depend on cell type or

a more complex single uptake mechanism involving both pathways (80). However, unlike other groups, Russell *et al.* could not decrease invasion with microfilament (cytochalasin D) or microtubule (vincristine and vinblastine) inhibitors (104). They suggested that cytoskeletal dependent endocytosis may be cell line specific or that high concentrations of inhibitors used in some studies could non-specifically inhibit other mechanisms (104).

Oelschlaeger *et al.* reported that *C. jejuni* uptake required coated-pit formation since invasion was inhibited greater than 98% by two different coated pit inhibitors (g-strophantin and monodansylcadaverine) (80). However, Russel *et al.* reported that coated pits were not required for invasion since the same two inhibitors did not alter *C. jejuni* invasion. Russell *et al.* also observed saturable time- and dose- dependent bacterial binding that is consistent with receptor mediated or transport limiting mechanism of invasion in Caco-2 cells (104). Work by Konkel at al. supported these findings by demonstrating that dansylcadaverine, a compound which disrupts receptor cycling, inhibited invasion (54). Inside the cell, *C. jejuni* is located within membrane-bound vacuoles (58, 104). Inhibitors of endosome acidification have no impact on *C. jejuni* intracellular survival (80).

Occasional rounding of Hep-2 cells was observed during a 6 hour incubation with *C. jejuni* (55). Prolonged incubation in the absence of antibiotics, led to the deterioration of *C. jejuni* infected cell monolayers (54). Cell monolayers infected with *C. jejuni* have been reported to show cell rounding, loss of adherence, and death after 24-48 hours (55). *C. jejuni* infection in infant *Macaca mulatta* also showed damaged epithelial cells which exhibited premature apoptosis (106). These observations suggest that intracellular *C. jejuni* may induce programmed cell death or produce cytotoxic compounds.

C. jejuni has also been located extracellularly in the mucosa and submucosa and extraintestinal complications and bacteremia have been reported (58, 106). It has been proposed that disease may involve translocation of *Campylobacter* across the intestinal epithelium resulting in tissue damage and inflammation (53). *C. jejuni* has been observed to translocate through and between Caco-2 cells (58). Inhibition of protein synthesis with

chloramphenicol reduced bacterial translocation. At 4°C, none of the organisms passed through the cells and very little adhered. The authors also observed a reduction of transcytosis at 20°C speculating that Caco-2 cell endocytic pathways are involved since eukaryotic endocytic and phagocytic processes are shown to be specifically inhibited at 18-22°C (58). However, the importance of *C. jejuni* cell translocation in disease needs further investigation.

1.2.4. INTRODUCTION TO FLAGELLA

The bipolar unsheathed *Campylobacter* flagella range from 2.6-3.9 μm in length and approximately 21 nm in width (86). Flagella are immunodominant during *Campylobacter* infection. Convalescent human serum contains antibodies to the flagellar proteins, flagellin (126). Some reports indicate that antibodies against flagellin are associated with homologous protection (25). *C. jejuni* has two genes coding for flagellin, *flaA* and *flaB*, which display 92.8% identity (3). The genes are arranged head-to-tail in the same direction separated by 174 bp. Each gene contains its own promoter: σ28 for *flaA* and σ54 for *flaB*. In wild-type organisms, *flaB* transcripts are not detected and *flaB* can be inactivated without a loss of motility (79). However, both *flaA* and *flaB* are detected by DNA hybridization analysis (118). In contrast, *flaA* mutants are relatively nonmotile and produce truncated flagella composed of only *flaB*. Perhaps *flaB* flagella are shorter because *flaB* is expressed at lower levels than *flaA* or the minor sequence differences in *flaB* result in inappropriate assembly of the filament (130). Organisms without *flaA* and *flaB* are aflagellate and non-motile. Wassenaar *et al.* suggested that expression of the two genes in *C. jejuni* is regulated at the transcriptional level so that predominantly one gene is transcribed at a time (129). Gene duplication in *Campylobacter* may suggest that either recombination between two genes occurs or that differential expression of the two genes, each with its own promoter, may lead to different flagellin types (129).

The flagellar organelle consists of an external filament, a hook and a basal body (79). Flagellin subunits are transported through the center of the filament and polymerize at the tip, during assembly of the flagellum. *flaA* and *flaB* sequences are homologous at both termini since these regions are needed for filament formation (transport, assembly, and stability) (3, 79). Since a consensus sequence for transport or assembly has not been found, Nuijten *et al.* suggest that the signal may be in conformation of flagellin (79). The overall structure of the flagellin genes is similar to the flagellins from the members of the *Enterobacteriaceae* (2, 3). The terminal regions are conserved while the central region displays differences in size and amino acid content. The central region of the flagellin genes is responsible for antigenic diversity encoding the H-antigen specificity of the *Enterobacteriaceae.*

Expression of *Campylobacter* flagellin genes is subject to phase and antigenic variation and is environmentally regulated. *C. jejuni* reverts between flagellated (fla+) and unflagellated (fla-) phenotypes. *In vitro*, Caldwell *et al.* observed changes from fla+ to fla- at a rate of $3.1X10^{-3}$ to $5.9X10^{-3}$ per cell per generation while fla- to fla+ reversion occurred at a rate of $4.0X10^{-7}$ to $8.0X10^{-7}$ per cell per generation (17). Aguero-Rosenfeld observed different reversion rates but the trend was still the same. *In vitro*, reversion from fla+ to fla- occured with a frequency of 9.2×10^{-6} per cell per generation but reversion in other direction could not be detected (1). However, *in vivo*, the excretion of fla+ phenotypes in stools is favored (see 1.2.5).

Campylobacter also has the ability to express flagella which differ antigenically. The calculated molecular weights of *C. jejuni* FlaA and FlaB are 59,538 Da and 59,909 Da respectively but, western blots have shown two bands of flagellin proteins at 62 kDa and 60 kDa (79, 118). The two bands do not represent products of *flaA* and *flaB*. It is speculated that posttranslational modification may be the cause of the difference between the calculated and experimental molecular weights and the presence of two flagellin bands both being the product of one gene, *flaA* (79). Interestingly, *E. coli* is unable to express

Campylobacter flagella which may be due to the lack of similar posttranslational modification mechanisms. The flagellar filament of *C. coli* is also composed of two flagellin subunits, FlaA and FlaB, whose antigenic specificities result from posttranslational modifications (see 1.2.6) (97). Also, it has been demonstrated that the *C. coli* σ54 promoter is subject to environmental regulation (4). The promoter is affected by the pH of the growth medium, the growth temperature and the concentration of certain inorganic salts and divalent cations (4). It is speculated that the *C. jejuni* flagellin promoters are also subject to enviromental regulation.

1.2.5. FLAGELLA IN ADHERENCE AND INVASION

Initially it was believed that flagella played an important role in adhesion (66). Several groups later demonstrated that flagella do not have adhesive roles (24, 70, 71, 127, 130). However, McSweegan *et al.* speculated that effective colonization was probably due to an interplay between motility, chemotaxis and adhesion (66). Mucus penetration is definitely facilitated by the spiral shape and darting motility of the organism. Also, Hugdahl *et al.* suggested that the positive chemotactic response generated by L-fucose, bile and mucin may influence colonization (45).

Nachamkin *et al.* examined the role of *C. jejuni* flagella as colonization factors for chicks (75). When chicks were infected with a wild-type strain, a non-flagellated, non-motile mutant and a partially motile mutant with truncated flagella, only the fully motile wild-type *C. jejuni* colonized the chicken ceca. In the hamster model described earlier, the authors observed a significant difference in the ability of wild-type *C. jejuni* to colonize hamsters compared with non-motile non-flagellated *C. jejuni* mutants (1). Also, as mentioned above, some hamsters challenged with the non-motile non-flagellated mutant excreted only wild-type Campylobacters in their feces (1). Human volunteers given both motile and nonmotile *C. jejuni* excreted only motile bacteria (129). Passage through the rabbit intestine also favored the fla+ phenotype (17). Similar observations were made

when mice were fed mixtures of fla+ and fla- bacteria, only fla+ bacteria were isolated from the stools (126). These results suggest that not only is motility necessary for colonization, but that the fla+ phenotype is selected for *in vivo*.

Grant *et al.* reported that flagella are not involved in adherence but flagellin and/or motility play a role in invasion (35). Since only *flaA* is expressed under usual *in vitro* conditions the authors suggested that either motility or the structural attributes of the *flaA* gene product were required for translocation across polarized epithelial cells. The finding that antibodies against flagella have no effect on adherence further suggests that flagella are not involved in adherence. They suggested that the flagellar structure may play a role in invasion.

In another experiment, a series of kanamycin resistant insertional mutants were generated and screened for invasion of INT 407 cells (136). One non-adherent non-invasive mutant had an insertion into the *flaA* gene which resulted in reduced motility and a truncated flagella. Two other adherent non-invasive mutants had full length flagella but were non motile. Since the two mot- inv- mutants expressed the full length *flaA* product, it can be concluded that motility rather than *flaA* is necessary for invasion. Since mutants with the *flaA* immobilized filament were still capable of adhering, other factors are probably involved in the motility-dependent invasion.

The general conclusion from these results is that aflagellate bacteria do not colonize *in vivo* and are less invasive *in vitro* (129).

1.2.6. FLAGELLAR ACCESSORY PROTEINS

The rotation of the flagellar filament is driven by a complex motor located within the cell envelope. Coupling between the filament and the motor is mediated through a structure known as the flagellar hook. The *Campylobacter* flagellar hook protein has an apparent molecular weight of 92.5-94 kDa (96). The hook is one of the longest and the largest described in the literature. The authors speculate that a large hook may be needed for

organisms with polar flagella or perhaps the size of the hook is a reflection of the viscous environments Campylobacters often encounter (96). Since the hook protrudes from the surface of the bacterial cell it can also contribute to the antigenic variation observed in *Campylobacter* spp. Interestingly, one class of surface-exposed hook epitopes was shared with serospecific flagellin epitopes and may involve posttranslational modification (96).

The possibility of posttranslational modifications and the involvement of these modifications in the antigenic variation among Campylobacters have been mentioned extensively throughout this introduction. Two genes have been identified that are required for posttranslational modification of the *C. coli* flagellin (39). Site-specific mutations in either gene caused a loss of antisera reactivity. Also, a mutation of one of the genes caused a shift in the mobility of the flagellin subunit in SDS-PAGE. In a rabbit model, infection with a *C. coli* strain containing a mutation in one gene showed a reduced ability to elicit protection against challenge. Guerry *et al.* suggested that surface-exposed posttranslational modifications may play a significant role in the protective immune response to *Campylobacter* infection and that posttranslational modifications are involved in the antigenic variation of *Campylobacter* flagella (39). The flagellins of *C. jejuni*, *C. coli*, and *C. fetus* are glycosylated (25). It was demonstrated that the flagellins contained terminal sialic acid residues (25). This is the first report of glycosylated eubacterial flagellins and the first report of sialylation of flagellin. Although sialic acid has also been detected in several *Campylobacter* LPS chemotypes (see 1.2.11 and Chapter 5), the genes identified in this study were not involved in LPS sialylation (39). The authors suggest that surface-exposed modifications on flagellin may be more important than amino acid sequences in eliciting protective immunity or that sialic acid residues may block antibody productions against amino acids (39).

Recently a 78,864 Da *C. jejuni* homolog, called FlhA, of the LcrD/FlbF family was identified (67). This family of proteins include LcrD of *Yersinia pestis*, MxiA of *Shigella flexneri*, and InvA of *S. typhimurium* which all play roles in invasion. The family also

includes FlbF of *Caulobacter crescentus* and FlhA of *E. coli* which play roles in flagellar expression, secretion, or assembly. In general, all LcrD/FlbF proteins are integral inner membrane proteins with roles in regulation or secretion of surface or extracellular proteins. The *C. jejuni* FlhA protein shared the most homology with the *C. crescentus* FlbF protein. An insertional mutant in the *flhA* gene did not synthesize flagella and was nonmotile (67). The *flhA* gene of *C. jejuni* is suggested to encode a protein which may play a yet unidentified role in flagellar biosynthesis.

As mentioned above, several kanamycin resistant insertional mutants were generated and screened for invasion of INT 407 cells (136). Two of the mutants were adherent but non-invasive with full length non-motile flagella. These two mutants have overlapping deletions in the same gene called *pflA* (paralysed flagella). The *pflA* gene encodes a protein with a predicted molecular weight of 90,977 kDa. The PflA protein was not homologous to any known proteins. The protein profile suggests that PflA may be an integral membrane protein. Also, all three motility mutants (including the non-adherent, non-invasive mutant with an insertion into the *flaA* gene resulting in reduced motility and truncated flagella) showed a structural change at the flagellar attachment site.

1.2.7. PILI

Recently, an environmentally regulated *Campylobacter* pilus-like appendage was identified (26). Electron micrographs of *C. jejuni*, *C. coli*, and *C. fetus* showed peritrichous pilus-like appendages when the bacteria were grown in bile salts. Piliated organisms resulted in a highly aggregative phenotype. The pili frequently formed bundles and were flexible rather than straight. Interestingly, the non-piliated mutant showed no reduction in adherence or invasion of INT 407 cells. The mutant also colonized ferrets but showed significantly reduced disease symptoms. However, it is possible that the mutated gene product may have other cellular functions or the mutation may have resulted in polar effects so that the reduction in virulence may not be solely due to non-piliation. The

authors have not yet purified the pilin subunit. Since *Campylobacter* can grow in high concentrations of bile, production of pili may be turned on *in vivo* and turned off *in vitro*. The authors speculate that the pili would be synthesized only after *C. jejuni* has successfully colonized the gastrointestinal tract. They also suggest that bile salts may serve as general inducers of virulence determinants of enteropathogens.

1.2.8. CONTROVERSIAL TOXINS

In a recent review, Sears and Kaper discussed three major *C. jejuni* toxins (heat labile enterotoxin, cytotoxin, and cytolethal distending toxin (CLDT)) (111). Several reports of *C. jejuni* toxin production can be found in the literature. However, there is currently no association between toxin production and clinical disease. Also, antibody responses to *C. jejuni* toxins are usually negative.

Previous reports demonstrated that culture supernatants from a portion of *C. jejuni* and *C. coli* isolates from humans contained cytotoxic activity. However the significance of the low titers of cytotoxin activity produced *in vitro* remained unclear. Cover *et al.* also detected cytotoxic activity in fecal filtrates but, the titers of cytotoxic activity in fecal filtrates from patients with *Campylobacter* enteritis, patients with diarrhea from other causes and healthy asymptomatic persons were not significantly different (22). They concluded that *Campylobacter* enteritis is not solely a cytotoxin mediated disease. Coote *et al.* detected low levels of cytotoxin activity, relative to the *E. coli* verotoxin 2 (VT-2) control, in *C. jejuni* cell extracts but not in supernatants (20). They did not observe agglutination activity with anti-cholera toxin (CT) antibody in cell extracts or supernatants. Coote *et al.* concluded that they had identified cell-associated cytotoxin activity. Perez-Perez *et al.* analysed the human immune response to potential toxins and looked for toxin activity in ELISA and cell culture assays (91). Their findings indicated that the production of a cholera-like toxin and cytotoxin by *C. jejuni* in the USA occurs in a few strains however a host immune response was absent. Johnson and Lior demonstrated that toxin producing

strains were frequently encountered among both human and nonhuman strains of *C. jejuni*, *C. coli*, and *C. laridis* (47). They were able to partially neutralize the *C. jejuni* enterotoxin with antibodies against cholera toxin and *E. coli* heat-labile enterotoxin (LT). However, they could not neutralize the *C. jejuni* cytotoxin with antibodies against the *Clostridium difficile* cytotoxin or *E. coli* verotoxin. Also, Vero and CHO cells treated with *C. jejuni* supernatants exhibited low levels of cyclic AMP (cAMP) as compared to cells treated with CT or *E. coli* labile toxin (47).

A 60-70 kDa *C. jejuni* enterotoxin was reported to induce fluid accumulation in rat and rabbit ileal loops, increase the permeability in rabbit skin, elongate CHO cells, bind GM1 gangliosides, be inhibited by anti-CT antibody, possesses a B-subunit similar to CT, and have enhanced expression in iron-enriched growth medium (126). Ruiz-Palacios *et al.* used a GM1 ELISA to identify enterotoxin producing Campylobacters (101). They demonstrated a strong association between toxin production and illness in that isolates from symptomatic children produced more toxin than isolates from asymptomatic children. However, they did not find an association between enterotoxin production and diarrhea.

Considerable evidence suggests that a cholera-like enterotoxin does not exist (69). Studies with DNA probes for CT and LT genes could not demonstrate homology at the molecular level (118). Everest *et al.* reported that *C. jejuni* infection in the rabbit ileal loop (RILT) model caused inflammatory reactions (29). However, they could not detect toxin activity in tissues or fluids of loops. Several investigators have reported that they were unable to detect toxin production (54). If enterotoxin production was detected, activity was much lower than that described for CT or LT. Also, patients with *C. jejuni* enteritis are not usually as dehydrated as are patients with secretory diarrhea caused by *V. cholerae* (47). In developed countries, the common response to *C. jejuni* infection is inflammatory diarrhea, not secretory diarrhea. However, Perez-Perez *et al.* examined *C. jejuni* isolates from Thailand, where secretory diarrhea is more common, as well as isolates from the USA (93). In the study, CT-like toxin could not be demonstrated by ELISA, cell culture assays,

GM1 binding assays, use of various growth conditions, or hybridization at low stringency with LT (93). Also, none of the *C. jejuni* infected patients showed seroconversion to CT.

Haemolysin proteins have also been identified which demonstrated a spectrum of activity against erythrocytes from different animals (44). However there have been no further reports characterizing these toxins.

1.2.9. CYTOLETHAL DISTENDING TOXIN

The cytolethal distending toxin (CLDT) was first described by Johnson and Lior in 1988 (46). They demontrated that the activity was heat sensitive, trypsin sensitive and nondialyzable. The cytolethal toxin activity (distension and death) was demonstrated in *Campylobacter* culture filtrates against CHO, Vero, HeLa, and Hep-2 cells. Over 40% of the isolates tested were positive for CLDT activity.

Recently, the CLDT genes were cloned and sequenced from *C. jejuni* 81-176 (95). Three genes encoding proteins of approximately 30 kDa (CdtA), 29 kDa (CdtB), and 21 kDa (CdtC) were identified. Most of the *C. jejuni* strains analysed produced significantly higher levels of CLDT than *C. coli*. Hybridization and PCR studies showed the probable presence of CdtB in all *Campylobacter* species tested. The *C. jejuni* CLDT is similar to *E. coli* CDT which is also encoded by three genes which produce proteins of similar size and probably identical functions. However, the amino acid sequences do not show high sequence homology. The initial reports suggested that only certain *Campylobacter* strains make toxin but the authors report that most *C. jejuni* strains make the toxin although in varying amounts. They suggest that it is too early to speculate whether high toxin production is associated with *C. jejuni* strains that cause diarrhea.

1.2.10.SECONDARY MESSENGER INDUCTION

The mechanisms of fluid secretion in *C. jejuni* infection remain unclear but inflammatory infiltrates and villous damage in association with fluid accumulation have

been shown (111). *C. jejuni* infection in a rabbit ileal loop (RILT) model caused inflammatory reactions (29). Since toxin was not detected in tissues or fluids of loops the authors proposed that host-derived mediators of secretion were important in disease. Everest *et al.* based their conclusion on previous observations that there were no obvious associations between toxin production and type of clinical illness and that antibodies against enterotoxin are not reported in convalescent serum. They also demonstrated that the host inflammatory mediator prostaglandin E2 (PGE2) elevates tissue cAMP in rabbit ileal loops suggesting active secretion is stimulated in acute and chronic inflammation of the intestine (28). Everest *et al.* also observed elevated levels of cAMP, PGE2, and leukotriene B4 in *C. jejuni*-infected rabbit ileal loops suggesting that released mediators may be needed for secretion (28). In another experiment, *C. jejuni* induced fluid secretion in the rat ileum (50). Addition of 100 μM verapamil and protein kinase C (PKC) antagonist H-7 significantly reduced fluid secretion. However, the calcium ionophore A23187 and PKC activator PMA (phorbol-12-myristate-13-acetate) did not enhance *C. jejuni* induced fluid accumulation. The calcium/calmodulin antagonist W-7 also had no effect. The authors suggested that increased PKC activity due to increased calcium uptake may be involved in secretion (50).

1.2.11.LIPOPOLYSACCHARIDE (LPS)

Non-enteric mucosal bacteria, usually lacking the O-oligosaccharide repeats, have outer membrane glycolipids known as lipooligosaccharides (LOS). The LOS of bacteria such *as Neisseria gonorrhoeae* and *Bordetella pertussis* are serologically different due to variations in the core oligosaccharide (37). Enteric bacteria that live in bile-rich environments have long hydrophilic sugar repeats known as LPS (37). The LPS of the family *Enterobacteriaceae*, as well as *Campylobacter*, consists of lipid A, core oligosaccharide, and O-polysaccharide chains. Typical members of this family such as *Salmonella* with a single core and *Escherichia coli* with five core regions exhibit more

conserved core oligosaccharide regions in their LPS (7). Although LPS are larger, LOS have been shown to be more structurally and antigenically diverse than LPS (37).

Lipid A is similar in structure and composition among the family *Enterobacteriaceae* (92). The LPS of Campylobacters share lipid A antigenic determinants with core regions of LPS from several other gram-negative organisms (92). The cross-reacting antibodies could explain why normal human serum contains bactericidal antibodies to *C. jejuni*. The LPS of *Campylobacter* is antigenically diverse resulting in a large number of serotypes (126). This observation has proven to be very useful in the heat stabile serotyping scheme of Penner and Hennessy (90). However, unlike other enteric bacteria, *C. jejuni* has LPS rich in sialiated glycans. Possible bacterial mechanisms used to avoid the host immune response may involve inhibiting antibody binding by the unusual sialylation of LPS (see Chapter 5) or the antigenic variability of LPS (77).

Analysis of the LPS from *C. jejuni* isolates from two siblings with GBS demonstrated that the LPS possessed the same O-chain structures linked to core oligosaccharides with different molecular weights (5). The study demonstrated ladder-like bands of similar periodicity but with slightly different mobilities on SDS-PAGE. These results suggested that identical O-chains may be linked to different core regions. Also, the *C. jejuni* core regions have been shown to mimic human glycosphingolipids (see Chapter 5). The molecular mimicry and the structural variability of the *C. jejuni* core oligosaccharide suggests that *C. jejuni* LPS is similar to LOS (6, 7).

Initially it was believed that *C. jejuni* LPS possessed adhesive properties. However, Moser and Hellmann reported that LPS did not have significant adhesive properties (70). They later showed that LPS-specific monoclonal antibodies could not inhibit binding, although LPS binding was detected with the antibodies, indicating that LPS is associated with, but is not a binding structure (71).

Although *Campylobacter* LPS is now generally believed not to be involved in adhesion, LPS has been shown to play several important roles: O-serotyping (heat-stable),

variation in antigenicity, and possible involvement in initiating Guillian-Barré syndrome. The association between *C. jejuni* LPS and GBS induction will be discussed in greater detail in Chapter 5.

1.2.12 POTENTIAL VIRULENCE PROTEINS

In addition to the flagellar proteins (1.2.4), flagellar accessory proteins (1.2.6), toxins (1.2.8 and 1.2.9), adhesion proteins (1.2.2), MapA (1.1.11) and lipid hydrolysing proteins (Chapter 4), which are described in other sections, there are several other proteins that may be potentially involved in virulence.

Bacteria produce high affinity iron scavenging systems to obtain iron from the limited-iron environment of the host. The low level of free iron in the host plays an important role in inhibiting bacterial growth. The *C. jejuni* ferric uptake regulatory (*fur*) gene was cloned and sequenced (64, 134). The *fur* gene encodes a protein with a predicted molecular weight of 18 kDa (64). *C. jejuni* synthesizes new outer membrane proteins in response to iron stress (134). It is speculated that an iron regulatory system may regulate a portion of *C. jejuni* virulence-associated genes. Evidence of iron-regulation of virulence factors has also been suggested by others (see below, (114)).

C. jejuni was shown to possess a gene encoding a 25 kDa superoxide dismutase (SOD) protein which would play a protective role against oxidative damage (94). The *sod* gene is most similar to SODs containing iron cofactors (FeSODs) so it was named SodB. *C. jejuni* produced at least 5 electrophoretically distinct bands of SOD activity. However it is believed that the cloned *sodB* gene is the only *sod* gene *C. jejuni* contains. If the multiple bands on gel are not resulting from multiple *sodB* genes, the closely migrating bands may be representative of some posttranslational modification. Interestingly, the SOD activity on gels of *E. coli* extracts was different suggesting that the same modification is not occuring in *E. coli*. The ability of the *C. jejuni* 81-176 *sodB* mutant strain to survive INT 407 cell invasion was significantly lower compared to the parent. This result suggests that

SodB may play a role in *C. jejuni* invasion of INT 407 cells or in intracellular survival. It is also interesting to note the identification of a potential *fur* binding site near the *sodB* gene.

A gene encoding catalase activity known as *katA* was cloned and sequenced (36). The gene encodes a protein with a predicted molecular weight of 58 kDa. Evidence suggests that KatA is structurally and enzymatically similar to hydrogen-peroxidases from other bacterial species. The authors suggest that the hydrogen peroxidase activity identified is the only catalase *C. jejuni* contains. A *katA* mutant was more sensitive to killing by hydrogen peroxide. The region upstream from *katA* also exhibits homology to the *fur* binding site. Since *C. jejuni* is a microaerophilic organism, this suggests that *C. jejuni* cannot grow in air due to its sensitivity to toxic products of oxygen metabolism. However, contaminated food is the main vehicle of *C. jejuni* infection suggesting that the organism can survive in air for a certian period of time. Also, *C. jejuni* has been previously shown to be aerotolerant and to adapt to an aerobic metabolism. Superoxide dismutase and catalase activity probably allows *C. jejuni* to survive in the environment. The activities may also assist in survival during macrophage respiratory burst.

The arylsulfatase gene, *astA*, has recently been identified (137). The gene encodes a protein with a predicted molecular weight of 69 kDa and with no sequence similarity to other known arylsulfatases. The degradative enzymes are suggested to play a role in periodontal disease caused by the *Campylobacter-Wolinella* family of oral bacteria. The possibility that this enzyme may be involved in virulence in enteropathogenic Campylobacters is currently being investigated in ferret animal models of diarrheal disease.

Recently an immunoreactive 18 kDa protein known as Omp18 was identified (14, 57). The mature Omp18 protein has an apparent molecular mass of 18 kDa which is higher than the calculated molecular mass. The discrepancy may be due to posttranslational modifications.

The Omp18 protein is homologous to peptidoglycan-associated lipoproteins (PALs) from other gram-negative bacteria. The Omp18 sequence also contains a peptidoglycan-associated alpha-helical consensus motif present in other PALs. The most abundant lipoprotein in *E. coli* is Braun's lipoprotein. Braun's lipoprotein is covalently attached to the peptidoglycan and is involved in maintenance of the cell envelope. PALs are less abundant proteins found in close association with but not covalently attached to the peptidoglycan. PAL may also function to maintain the cell envelope since *E. coli* PAL mutants exhibit sensitivity to detergent and EDTA and leak periplasmic contents. Since Braun's lipoprotein was absent from outer membrane profiles of *C. jejuni*, PAL may play an important structural role in the maintenance of the *C. jejuni* envelope (63).

PALs have been shown to be highly immunogenic and conserved among different gram-negative bacteria. Omp18 may be a potential vaccine candidate for a multisubunit vaccine. Omp18 is also a good candidate as an antigen for serological diagnosis of past *C. jejuni* infections (14). The development of a reliable marker for past *C. jejuni* infection is important for epidemiological studies and for revealing the role of *C. jejuni* in causing extraintestinal complications such as Guillain-Barré syndrome.

A summary of all identified proteins which may play a role in virulence is presented in Table 1.1.

TABLE 1.1. PROTEINS POTENTIALLY INVOLVED IN VIRULENCE

Protein	Proposed Function	MW (kDa)	Cellular Location	Reference
FlaA	flagellin	59.5	surface	(79)
FlaB	flagellin	60	surface	(79)
PflA	flagellar motility / structure	91	integral memb	(136)
	flagellin hook protein	92.5-94	surface	(96)
FlhA	flagellar biosynthesis	79		(67)
	pilus		surface	(26)
	CT-like enterotoxin	60-70		(126)
CdtA	cytolethal distending toxin	30		(95)
CdtB	cytolethal distending toxin	29		(95)
CdtC	cytolethal distending toxin	21		(95)
	haemolysin		periplasmic	unpublished
Peb1	amino acid binding protein bacterial adherence	28	surface	(87-89)
Peb2	no homologs	29	surface	(88, 89)
Peb3	homology to pili or enterotoxin	30	surface	(88, 89)
Peb4	protein transformation	31	periplasmic	(15, 88, 89)
MapA	membrane associated protein	22	IM lipoprotein	(116)
Fur	ferric uptake regulatory gene	18		(64, 134)
KatA	catalase	58		(36)
AstA	arylsulfatase	69		(137)
Omp18	peptidoglycan associated	18	OM lipoprotein	(14, 57)
SodB	iron superoxide dismutase	25		(94, 98)
	lipase chaperone	14	membrane	Chap. 4
	lipase		membrane	Chap. 4
	phospholipase C	14 and/or 55	membrane	Chap. 4
	phospholipase A_2		cell sonicate	Chap. 4, (61)

1.3. HYPOTHESIS AND OBJECTIVES

It is evident from the introduction that there remains much to be learned about how *Campylobacter jejuni* causes disease. Therefore, we were interested in studying the pathogenic mechanisms of *C. jejuni*. We believed that by taking a holistic approach we would gain a better understanding of *C. jejuni* pathogenesis.

When *C. jejuni* is ingested by the host, the organisms are transported down the gastrointestinal tract by peristaltic waves. Once in the stomach, gastric acid has been suggested to act as a barrier to *Campylobacter* infection (see section 1.1.5, (13)). Therefore, *Campylobacter* is moved along to the small intestine by peristalsis. *Campylobacter* viability is not affected by the pH of the intestine or by the presence of bile (see section 1.1.5, (13)). We were first interested in examining the effect of pH on *Campylobacter* motility (Chapter 2). If the organisms are motile in the small intestine, they will be confronted with the task of moving across the mucus blanket lining the intestinal epithelial cells. Also, penetration of the mucus blanket must occur quickly if the organisms want to avoid elimination by the flowing action of the intestinal mucus. In Chapter 2, we calculated *C. jejuni* swimming velocities in solutions with viscosities similar to that of mucus. Once the organisms penetrate the mucus successfully, they will then have to bind to intestinal epithelial cells and induce non-professional phagocytosis by these cells. We examined the effect of viscosity on *Campylobacter* attachment and invasion of CaCo-2 cells (Chapter 2). We also looked at signal pathways that may be involved in *C. jejuni* binding and invasion *in vitro* (Chapter 6). To further investigate how *C. jejuni* interacts with intestinal cells, we looked for host cell receptors and for *C. jejuni* adhesins (Chapter 3). During the infection process, *Campylobacter* induces several physiological changes in the host through the action of various bacterial virulence factors. Characterization of *C. jejuni* proteins, which interact with the host, led to the identification of several *C. jejuni* proteins that may play a role in virulence (Chapter 4). One of these proteins exhibited characteristics similar to a protein family in which one of its members has been implicated

in the induction of GBS. Since *C. jejuni* has recently been shown to be a precipitating factor in the initiation of GBS, we were interested in examining whether this protein plays a role in GBS induction (Chapter 5).

Several organisms were used in the following chapters. A summary of the organisms used, the chapter in which they appear, and the investigator or organization that provided the particular strains is shown in Table 1.2.

TABLE 1.2. SUMMARY OF ORGANISMS USED IN STUDIES

Organism	Strain and/or Type	Phenotype / Isolation Year	Chapter	Source
Campylobacter jejuni	UA580 / Lior 4 / NTCC 11168	high passage, 1984	2, 3, 4, 5, 6	D. E. Taylor
Campylobacter jejuni	ER1109 / Lior 40	low passage, 1993	2, 4	Alberta Provincial Laboratory
Campylobacter jejuni	E863 / ND[a]	low passage, 1993	2, 4, 6	Alberta Provincial Laboratory
Campylobacter jejuni	81116 / Lior 6 / NTCC 11828	(82)	4	T. J. Trust
Campylobacter jejuni	UA581 / Lior 10	fla-mot-, 1984	2	D. E. Taylor
Helicobacter pylori	UA763 / NTCC 11639	1990	3	D. E. Taylor
Salmonella choleraesuis	SL2824	(78)	3	G. D. Armstrong
Salmonella enteritidis	710063	chicken isolate	2	M. Finlayson
Escherichia coli	O157:H7 / E32511	(110), 1983	2	G. D. Armstrong
Escherichia coli	JA221	(42)	4	S. T. Jorgensen

[a]ND = serotype not determined

1.4. REFERENCES

1. **Aguero-Rosenfeld, M. E., X.-H. Yang, and I. Nachamkin**. 1990. Infection of adult Syrian hamsters with flagellar variants of *Campylobacter jejuni*. Infect. Immun. **58:** 2214-2219.

2. **Alm, R. A., P. Guerry, M. E. Power, H. Lior, and T. J. Trust**. 1991. Analysis of the role of flagella in the heat-labile Lior serotyping scheme of thermophilic Campylobacters by mutant allele exchange. J. Clin. Microbiol. **29:** 2438-2445.

3. **Alm, R. A., P. Guerry, and T. J. Trust**. 1993b. Distribution and polymorphism of the flagellin genes from isolates of *Campylobacter coli* and *Campylobacter jejuni*. J. Bacteriol. **175:** 3051-3057.

4. **Alm, R. A., P. Guerry, and T. J. Trust**. 1993a. The *Campylobacter* σ54 *flaB* flagellin promoter is subject to environmental regulation. J. Bacteriol. **175:** 4448-4455.

5. **Aspinall, G. O., S. Fujimoto, M. A. G., H. Pang, L. A. Kurjanczyk, and J. L. Penner**. 1994. Lipopolysaccharides from *Campylobacter jejuni* associated with Guillain-Barré syndrome patients mimic human gangliosides in structure. Infect. Immun. **62:** 2122-2125.

6. **Aspinall, G. O., A. G. McDonald, H. Pang, L. A. Kurjanczyk, and J. L. Penner**. 1994. Lipopolysaccharides of *Campylobacter jejuni* serotype O:19: structures of core oligosaccharide regions from the serostrain and two bacterial isolates from patients with the Guillain-Barré syndrome. Biochem. **33:** 241-249.

7. **Aspinall, G. O., A. G. McDonald, T. S. Raju, H. Pang, L. A. Kurjanczyk, J. L. Penner, and A. P. Moran**. 1993. Chemical structure of the core region of *Campylobacter jejuni* serotype 0:2 lipopolysaccharide. Eur. J. Biochem. **213:** 1029-1037.

8. **Babakhani, F. K., G. A. Bradley, and L. A. Joens**. 1993. Newborn piglet model for Campylobacteriosis. Infect. Immun. **61:** 3466-3475.

9. **Bag, P. K., T. Ramamurthy, and U. B. Nair**. 1993. Evidence for the presence of a receptor for the cytolethal distending toxin (CLDT) of *Campylobacter jejuni* on CHO and HeLa cell membranes and development of a receptor-based enzyme-linked immunosorbent assay for detection of CLDT. FEMS Microbiol. Lett. **114:** 285-292.

10. **Baqar, S., N. D. Pacheco, and F. M. Rollwagen**. 1993. Modulation of mucosal immunity against *Campylobacter jejuni* by orally administered cytokines. Antimicrob. Agents Chemother. **37:** 2688-2692.

11. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser**. 1988. Experimental *Campylobacter jejuni* infection in humans. J. Infect. Dis. **157:** 472-479.

12. **Black, R. E., D. Perlman, M. L. Clements, M. M. Levine, and M. J. Blaser**. 1992. Human volunteer studies with *Campylobacter jejuni*, p. 207-215. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

13. **Blaser, M. J., H. L. Hardesty, B. Powers, and W. L. Wang**. 1980. Survival of *Campylobacter fetus* subsp. *jejuni* in biological milieus. J. Clin. Microbiol. **11:** 309-313.

14. **Burnens, A., U. Stucki, J. Nicolet, and J. Frey**. 1995. Identification and characterization of an immunogenic outer membrane protein of *Campylobacter jejuni*. J. Clin. Microbiol. **33:** 2826-2832.

15. **Burucoa, C., C. Frémaux, Z. Pei, M. Tummuru, M. J. Blaser, Y. Centatiempo, and J. L. Fauchère**. 1995. Nucleotide sequence and characterization of *peb4A* encoding an antigenic protein in *Campylobacter jejuni*. Res. Microbiol. **146:** 467-476.

16. **Butzler, J.-P., and J. Oosterom**. 1991. *Campylobacter*: pathogenicity and significance in foods. Int. J. Food Microbiol. **12:** 1-8.

17. **Caldwell, M. B., P. Guerry, E. C. Lee, J. P. Burans, and R. I. Walker**. 1985. Reversible expression of flagella in *Campylobacter jejuni*. Infect. Immun. **50:** 941-943.

18. **Chang, N., and D. E. Taylor**. 1990. Use of pulsed-field agarose gel electrophoresis to size genomes of *Campylobacter* species and to construct a *Sal*I map of *Campylobacter jejuni* UA580. J. Bacteriol. **172:** 5211-5217.

19. **Cinco, M., E. Banfi, E. Ruaro, D. Crevatin, and D. Crotti**. 1984. Evidence for L-fucose (6-deoxy-L-galactopyranose)-mediated adherence of *Campylobacter* spp. to epithelial cells. FEMS Microbiol. Letters. **21:** 347-351.

20. **Coote, J. G., and T. Arain**. 1996. A rapid, colourimetric assay for cytotoxin activity in *Campylobacter jejuni*. FEMS Immunol. Med. Microbiol. **13:** 65-70.

21. **Cover, T. L., and M. J. Blaser**. 1989. The pathobiology of *Campylobacter* infections in humans. Ann. Rev. Med. **40:** 269-285.

22. **Cover, T. L., G. I. Perez-Perez, and M. J. Blaser**. 1990. Evaluation of cytotoxic activity in fecal filtrates from patients with *Campylobacter jejuni* or *Campylobacter coli* enteritis. FEMS Microbiol. Lett. **70:** 301-304.

23. **Cowden, J.** 1992. *Campylobacter*: epidemiological paradoxes. BMJ. **305:** 132-133.

24. **deMelo, M. A., and J. Pechere**. 1990. Identification of *Campylobacter jejuni* surface proteins that bind to eucaryotic cells *in vitro*. Infect. Immun. **58:** 1749-1756.

25. **Doig, P., N. Kinsella, P. Guerry, and T. J. Trust**. 1996. Characterization of a post-translational modification of *Campylobacter* flagellin: identification of a sero-specific glycosyl moiety. Mol. Microbiol. **19:** 379-387.

26. **Doig, P., R. Yao, D. H. Burr, P. Guerry, and T. J. Trust**. 1996. An environmentally regulated pilus-like appendage involved in *Campylobacter* pathogenesis. Mol. Microbiol. **20:** 885-894.

27. **Doyle, M. P., and D. M. Jones**. 1992. Food-borne transmission and antibiotic resistance of *Campylobacter jejuni*, p. 45-48. *In* Nachamkin, I., M. J. Blaser, and L. S.

Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

28. **Everest, P. H., A. T. Cole, C. J. Hawkey, S. Knutton, H. Goosens, J.-P. Butzler, J. M. Ketney, and P. H. Williams**. 1993. Roles of leukotriene B_4, prostaglandin E_2, and cyclic AMP in *Campylobacter jejuni*-induced intestinal fluid secretion. Infect. Immun. **61:** 4885-4887.

29. **Everest, P. H., H. Goossens, P. Sibbons, D. R. LLoyd, S. Knutton, R. Leece, J. M. Ketley, and P. H. Williams**. 1993. Pathological changes in the rabbit ileal loop model caused by *Campylobacter jejuni* from human colitis. J. Med. Microbiol. **38:** 316-321.

30. **Fauchère, J.-L., M. Kervella, A. Rosenau, J. M. Pagès, and C. Fendri**. 1992. In vitro study of virulence factors of enteric *Campylobacter* spp., p. 168-175. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

31. **Fox, J. G.** 1992. *In vivo* models of enteric Campylobacteriosis: natural and experimental infections, p. 131-138. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

32. **Funke, G., R. Baumann, J. L. Penner, and M. Altwegg**. 1994. Development of resistance to macrolide antibiotics in an AIDS patient treated with clarithromycin for *Campylobacter jejuni* diarrhea. Eur. J. Clin. Microbiol. Infect. Dis. **13:** 612-615.

33. **Garcia, M. M.** 1990. *Campylobacter*: "the next *Salmonella*". Safety watch - Agriculture Canada. **15:** 1-2.

34. **Giesendorf, B. A. J., A. van Belkum, A. Koeken, H. Stegeman, M. H. C. Henkens, J. van der Plas, H. Goosens, H. G. M. Niesters, and W. G. V. Quint**. 1993. Development of species-specific DNA probes for *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* by polymerase chain reaction fingerprinting. J. Clin. Microbiol. **31:** 1541-1546.

35. **Grant, C. C. R., M. E. Konkel, J. W. Cieplak, and L. S. Tompkins**. 1993. Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. Infect. Immun. **6:** 1764-1771.

36. **Grant, K. A., and S. F. Park**. 1995. Molecular characterization of *katA* from *Campylobacter jejuni* and generation of a catalase-deficient mutant of *Campylobacter coli* by interspecific allelic exchange. Microbiol. **141:** 1369-1376.

37. **Griffiss, J. M.** 1995. The role of bacterial lipooligosaccharides in the pathogenesis of human disease. Trends Glycosci. Glycotech. **7:** 461-478.

38. **Griffiths, P. L.** 1993. Morphological changes of *Campylobacter jejuni* growing in liquid culture. Lett. Appl. Microbiol. **17:** 152-155.

39. **Guerry, P., P. Doig, R. A. Alm, D. H. Burr, N. Kinsella, and T. J. Trust**. 1996. Identification and characterization of genes required for post-translational modification of *Campylobacter coli* VC167 flagellin. Mol. Microbiol. **19:** 369-378.

40. **Guerry, P., P. M. Pope, D. H. Burr, J. Leifer, S. W. Joseph, and A. L. Bourgeois**. 1994. Development and characterization of *recA* mutants of *Campylobacter jejuni* for inclusion in attenuated vaccines. Infect. Immun. **1994:** 426-432.

41. **Harris, L. A., S. M. Logan, P. Guerry, and T. J. Trust**. 1987. Antigenic variation of *Campylobacter* flagellin. J. Bacteriol. **169:** 5066-5071.

42. **Hobson, A. H., C. M. Buckley, J. L. Aamand, S. T. Jørgensen, B. Diderichsen, and D. J. McConnell**. 1993. Activation of bacterial lipase by its chaperone. Proc. Natl. Acad. Sci. USA. **90:** 5682-5686.

43. **Høiby, N., M. T. Collins, F. Espersen, J. B. Hertz, G. E. Hoff, and P. O. Schiøtz**. 1987. Taxonomic application of crossed immunoelectrophoresis. Int. J. Syst. Bacteriol. **37:**

44. **Hossain, A., D. E. S. Stewart-Tull, and J. H. Freer**. 1993. Heat-labile and heat-stable haemolysins of *Campylobacter jejuni*. FEMS Immunol. Med. Microbiol. **6:** 331-340.

45. **Hugdahl, M. B., J. T. Beery, and M. P. Doyle**. 1988. Chemotactic behavior of *Campylobacter jejuni*. Infect. Immun. **56:** 1560-1566.

46. **Johnson, W. M., and H. Lior**. 1988. A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. Microbiol. Pathogen. **4:** 115-126.

47. **Johnson, W. M., and H. Lior**. 1986. Cytotoxic and cytotonic factors produced by *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter laridis*. J. Clin. Microbiol. **24:** 275-281.

48. **Jones, D. M., E. M. Sutcliffe, R. Rios, A. J. Fox, and A. Curry**. 1993. *Campylobacter jejuni* adapts to aerobic metabolism in the environment. J. Med. Microbiol. **38:** 145-150.

49. **Kapperud, G., J. Lassen, S. M. Ostroff, and S. Aasen**. 1992. Clinical features of sporadic *Campylobacter* infections in Norway. Scan. J. Infect. Dis. **24:** 741-749.

50. **Kaur, R., N. K. Ganguly, L. Kumar, and B. N. S. Walia**. 1993. Studies on the pathophysiological mechanism of *Campylobacter jejuni*-induced fluid secretion in rat ileum. FEMS Microbiol. Lett. **111:** 327-330.

51. **Kervella, M., J.-M. Pages, Z. Pei, G. Grollier, M. J. Blaser, and J.-L. Fauchere**. 1993. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. Infect. Immun. **61:** 3440-3448.

52. **Khakhria, R., and H. Lior**. 1992. Extended phage-typing scheme for *Campylobacter jejuni* and *Campylobacter coli*. Epidemiol. Infect. **108:** 403-414.

53. **Konkel, M. E., and W. Cieplak Jr.** 1992. Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalization. Infect. Immun. **60:** 4945-4949.

54. **Konkel, M. E., S. F. Hayes, L. A. Joens, and W. Cieplak Jr.** 1992a. Characteristics of the internalization and intracellular survival of *Campylobacter jejun*i in human epithelial cell cultures. Microbial Pathog. **13:** 357-370.

55. **Konkel, M. E., and L. A. Joens**. 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect. Immun. **57:** 2984-2990.

56. **Konkel, M. E., D. J. Mead, and W. Cieplak Jr.** 1993. Kinetic and antigenic characterization of altered protein synthesis by *Campylobacter jejuni* during cultivation with human epithelial cells. J. Infect. Dis. **168:** 948-954.

57. **Konkel, M. E., D. J. Mead, and W. Cieplak Jr.** 1996. Cloning, sequencing, and expression of a gene from *Campylobacter jejuni* encoding a protein (Omp18) with similarity to peptidoglycan-associated lipoproteins. Infect. Immun. **64:** 1850-1853.

58. **Konkel, M. E., D. J. Mead, S. F. Hayes, and W. Cieplak Jr.** 1992b. Translocation of *Campylobacter jejuni* across human polarized epithelial cell monolayer cultures. J. Infect. Dis. **166:** 308-315.

59. **Kuusela, P., A. P. Moran, T. Vartio, and T. U. Kosunen**. 1989. Interaction of *Campylobacter jejuni* with extracellular matrix components. Biochim. Biophys. Acta. **993:** 297-300.

60. **Lam, K. M., A. J. DaMassa, T. Y. Morishita, H. L. Shivaprasad, and A. A. Bickford**. 1992. Pathogenicity of *Campylobacter jejuni* for turkeys and chickens. Avian Dis. **36:** 359-363.

61. **Langton, S. R., and S. D. Cesareo**. 1992. *Helicobacter pylori* associated phospholipase A_2 activity: a factor in peptic ulcer production? J. Clin. Pathol. **45:** 221-224.

62. **Lindblom, G.-B., C. Åhrén, J. Changalucha, R. Gabone, B. Kaijser, L.-Å. Nilsson, E. Sjögren, A.-M. Svennerholm, and M. Temu**. 1995. *Campylobacter jejuni / coli* and enterotoxigenic *Escherichia coli* (ETEC) in faeces from children and adults in Tanzania. Scand. J. Infect. Dis. **27:** 589-593.

63. **Logan, S. M., and T. J. Trust**. 1982. Outer membrane characteristics of *Campylobacter jejuni*. Infect. Immun. **38:** 898-906.

64. **Loong-Chan, V., H. Louie, and H. L. Bingham**. 1995. Cloning and transcription regulation of the ferric uptake regulatory gene of *Campylobacter jejuni* TGH9011. Gene. **164:** 25-31.

65. **McSweegan, E., D. H. Burr, and R. I. Walker**. 1987. Intestinal mucus gel and secretory antibody are barriers to *Campylobacter jejuni* adherence to INT 407 cells. Infect. Immun. **55:** 1431-1435.

66. **McSweegan, E., and R. I. Walker**. 1986. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect. Immun. **53:** 141-148.

67. **Miller, S., E. C. Pesci, and C. L. Pickett**. 1993. A *Campylobacter jejuni* homolog of the LcrD/FlbF family of proteins is necessary for flagellar biosynthesis. Infect. Immun. **61:** 2930-2936.

68. **Millson, M., M. Bokhout, J. Carlson, L. Spielberg, R. Aldis, A. Borczyk, and H. Lior**. 1991. An outbreak of *Campylobacter jejuni* gastroenteritis linked to meltwater contamination of a municipal well. Can. J. Public Health. **82:** 27-31.

69. **Mishu-Allos, B., and M. J. Blaser**. 1995. *Campylobacter jejuni* and the expanding spectrum of related infections. Clin. Infect. Dis. **20:** 1092-1099.

70. **Moser, I., and E. Hellmann**. 1989. *In vitro* binding of *Campylobacter jejuni* surface proteins to murine small intestinal cell membranes. Med. Microbiol. Immunol. **178:** 217-228.

71. **Moser, I., W. F. K. J. Schröder, and E. Hellmann**. 1992. *In vitro* binding of *Campylobacter jejuni/coli* outer membrane preparations to INT 407 cell membranes. Med. Microbiol. Immunol. **180:** 289-303.

72. **Murphy Jr., G. S., P. Echeverria, L. R. Jackson, M. K. Arness, C. LeBron, and C. Pitarangsi**. 1996. Ciprofloxacin- and azithromycin-resistant Campylobacter causing traveler's diarrhea in U. S. troops deployed to Thailand in 1994. Clin. Infect. Dis. **22:** 868-869.

73. **Nachamkin, I., K. Bohachick, and C. A. Patton**. 1993. Flagellin gene typing of *Campylobacter jejuni* by restriction length polymorphism analysis. J. Clin. Microbiol. **31:** 1531-1536.

74. **Nachamkin, I., H. Ung, and C. M. Patton**. 1996. Analysis of HL and O serotypes of Campylobacter strains by the flagellin gene typing system. J Clin Microbiol. **34:** 277-281.

75. **Nachamkin, I., X.-H. Yang, and N. J. Stern**. 1993. Role of *Campylobacter jejuni* flagella as colonization factors for three-day-old chicks: analysis with flagellar mutants. Appl. Environ. Microbiol. **59:** 1269-1273.

76. **Newell, D. G., and H. McBride**. 1985. Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines. J. Hyg. Camb. **95:** 217-227.

77. **Newell, D. G., and I. Nachamkin**. 1992. Immune responses directed against *Campylobacter jejuni*, p. 201-206. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

78. **Nnalue, N. A., and B. A. D. Stocker**. 1986. Some *galE* mutants of *Salmonella choleraesuis* retain virulence. Infect. Immun. **54:** 635-640.

79. **Nuijten, P. J. M., F. J. A. M. van Asten, W. Gaastra, and B. A. M. van der Zeijst**. 1990. Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. J. Biol. Chem. **265:** 17798-17804.

80. **Oelschlaeger, T. A., P. Guerry, and D. J. Kopecko**. 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. Proc. Natl. Acad. Sci. **90:** 6884-6888.

81. **Oyofo, B. A., and D. M. Rollins**. 1993. Efficacy of filter types for detecting *Campylobacter jejuni* and *Campylobacter coli* in environmental water samples by polymerase chain reaction. Appl. Environ. Microbiol. **59:** 4090-4095.

82. **Palmer, S. R., P. R. Gully, J. M. White, A. D. Pearson, W. G. Suckling, D. M. Jones, J. C. Rawes, and J. L. Penner**. 1983. Water-borne outbreak of *Campylobacter* gastroenteritis. Lancet. **1:** 287-290.

83. **Panigrahi, P., G. Losonsky, L. DeTolla, and J. G. Morris Jr.** 1992. Human immune response to *Campylobacter jejuni* proteins expressed *in vivo*. Infect. Immun. **60:** 4938-4944.

84. **Patton, C. M., M. A. Nicholson, S. M. Ostroff, A. A. Ries, I. K. Wachsmuth, and R. V. Tauxe**. 1993. Common somatic O and heat-labile serotypes among *Campylobacter* strains from sporadic infections in the United States. J. Clin. Microbiol. **31:** 1525-1530.

85. **Patton, C. M., I. K. Wachsmuth, G. M. Evins, J. A. Kiehlbauch, B. D. Plikaytis, N. Troup, L. S. Tompkins, and H. Lior**. 1991. Evaluation of 10 methods to distinguish epidemic-associated *Campylobacter* strains. J. Clin. Microbiol. **29:** 680-688.

86. **Pead, P. J.** 1979. Electron microscopy of *Campylobacter jejuni*. J. Med. Microbiol. **12:** 383-385.

87. **Pei, Z., and M. J. Blaser**. 1993. PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems. J. Biol. Chem. **268:** 18717-18725.

88. **Pei, Z., R. T. Ellison III, and M. J. Blaser**. 1991. Identification, purification, and characterization of major antigenic proteins of *Campylobacter jejuni*. J. Biol. Chem. **266:** 16363-16369.

89. **Pei, Z., R. T. Ellison III, and M. J. Blaser**. 1992. Identification and characterization of major common antigens from *Campylobacter jejuni*, p. 236-237. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

90. **Penner, J. L., and J. N. Hennessy**. 1980. Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. J. Clin. Microbiol. **12:** 732-737.

91. **Perez-Perez, G. I., D. L. Cohn, R. L. Guerrant, C. M. Patton, L. B. Reller, and M. J. Blaser**. 1989. Clinical and immunologic significance of cholera-like toxin and cytotoxin production by *Campylobacter* species in patients with acute inflammatory diarrhea in the USA. J. Infect. Dis. **160:** 460-468.

92. **Perez-Perez, G. I., J. A. Hopkins, and M. J. Blaser**. 1986. Lipopolysaccharide structures in *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* are immunologically related to *Campylobacter* spp. Infect. Immun. **51:** 204-208.

93. **Perez-Perez, G. I., D. N. Taylor, P. D. Echeverria, and M. J. Blaser**. 1992. Lack of evidence of enterotoxin involvement in pathogenesis of *Campylobacter* diarrhea, p. 184-192. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

94. **Pesci, E. C., D. L. Cottle, and C. L. Pickett**. 1994. Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. Infect. Immun. **62:** 2687-2694.

95. **Pickett, C. L., E. C. Pesci, D. L. Cottle, G. Russell, A. Nalca-Erdem, and H. Zeytin**. 1996. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* genes. Infect. Immun. **64:** 2070-2078.

96. **Power, M. E., R. A. Alm, and T. J. Trust**. 1992. Biochemical and antigenic properties of the *Campylobacter* flagellar hook protein. J. Bacteriol. **174:** 3874-3883.

97. **Power, M. E., P. Guerry, W. D. McCubbin, C. M. Kay, and T. J. Trust**. 1994. Structural and antigenic characteristics of *Campylobacter coli* FlaA flagellin. J. Bacteriol. **176:** 3303-3313.

98. **Purdy, D., and S. F. Park**. 1994. Cloning, nucleotide sequence and characterization of a gene encoding superoxide dismutase from *Campylobacter jejuni* and *Campylobacter coli*. Microbiol. **140:** 1203-1208.

99. **Reed, R. P., I. R. Friedland, F. O. Wegerhoff, and M. Khoosal**. 1996. *Campylobacter* bacteremia in children. Pediatr. Infect. Dis. J. **15:** 345-348.

100. **Rollwagen, F. M., N. D. Pacheco, J. D. Clements, O. Pavlovskis, D. M. Rollins, and R. I. Walker**. 1993. Killed *Campylobacter* elicits immune response and protection when administered with an oral adjuvant. Vaccine. **11:** 1316-1320.

101. **Ruiz-Palacios, G. M., L. E. Cervantes, D. S. Newburg, Y. Lopez-Vidal, and J. J. Calva**. 1992. *In vitro* models for studying *Campylobacter* infections, p. 176-183. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. America Society for Microbiology, Washington, D. C. .

102. **Ruiz-Palacios, G. M., E. Escamilla, and N. Torres**. 1981. Experimental *Campylobacter* diarrhea in chickens. Infect. Immun. **34:** 250-255.

103. **Russell, R. G.** 1992. *Campylobacter jejuni* colitis and immunity in primates: epidemiology of natural infection, p. 148-157. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

104. **Russell, R. G., and J. D. C. Blake**. 1994. Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. Infect. Immun. **62:** 3773-3779.

105. **Russell, R. G., M. J. Blaser, J. I. Sarmiento, and J. Fox**. 1989. Experimental *Campylobacter jejuni* infection in *Macaca nemestrina*. Infect. Immun. **57:** 1438-1444.

106. **Russell, R. G., M. O'Donnoghue, J. D. C. Blake, J. Zulty, and L. J. DeTolla**. 1993. Early colonic damage and invasion of *Campylobacter jejuni* in experimentally challenged infant *Macaca mulatta*. J. Infect. Dis. **168:** 210-215.

107. **Saeed, A. M., N. V. Harris, and R. F. DiGiacomo**. 1993. The role of exposure to animals in the etiology of *Campylobacter jejuni / coli* enteritis. Am. J. Epidemiol. **137:** 108-114.

108. **Sánchez, R., V. Fernández-Baca, M. D. Díaz, P. Muñoz, M. Rodríguez-Créixems, and E. Bouza**. 1994. Evolution of susceptibilities of *Campylobacter* spp. to quinolones and macrolides. Antimicrob. Agents Chemother. **38:** 1879-1882.

109. **Schoeni, J. L., and A. C. L. Wong**. 1994. Inhibition of *Campylobacter jejuni* colonization in chicks by defined competitive exclusion bacteria. Appl. Environ. Microbiol. **60:** 1191-1197.

110. **Scotland, S. M., G. A. Willshaw, H. R. Smith, and B. Rowe**. 1987. Properties of strains of *Escherichia coli* belonging to serogroup O157 with special reference to production of Vero cytotoxins VT1 and VT2. Epidem. Inf. **99:** 613-624..

111. **Sears, C. L., and J. B. Kaper**. 1996. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. Microbiol. Rev. **60:** 167-215.

112. **Siddique, A. B., and S. Q. Akhtar**. 1991. Study on the pathogenicity of *Campylobacter jejuni* by modifying the medium. J. Trop. Med. Hyg. **94:** 175-179.

113. **Skirrow, M. B., and M. J. Blaser**. 1992. Clinical and epidemiologic considerations, p. 3-8. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

114. **Sokol, P. A., and D. E. Woods**. 1984. Relationship of iron and extracellular virulence factors to *Pseudomonas aeruginosa* lung infections. J. Med. Microbiol. **18:** 125-133.

115. **Stern, N. J.** 1992. Reservoirs for *Campylobacter jejuni* and approaches for intervention in poultry, p. 49-60. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

116. **Stucki, U., J. Frey, J. Nicolet, and A. P. Burnens**. 1995. Identification of *Campylobacter jejuni* on the basis of a species-specific gene that encodes a membrane protein. J. Clin. Microbiol. **33:** 855-859.

117. **Taylor, D. E.** 1992. Antimicrobial resistance of Campylobacter jejuni and Campylobacter coli to tetracycline, chloramphenicol, and erithromycin, p. 74-86. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

118. **Taylor, D. E.** 1992. Genetics of *Campylobacter* and *Helicobacter*. Annu. Rev. Microbiol. **46:** 35-64.

119. **Taylor, D. E., and N. Chang**. 1987. Immunoblot and enzyme-linked immunosorbent assays of *Campylobacter* major outer-membrane protein and application to the differentiation of *Campylobacter* species. Mol. Cell. Probes. **1:** 261-274.

120. **Taylor, D. N., D. M. Perlman, P. D. Echeverria, U. Lexomboon, and M. J. Blaser**. 1993. *Campylobacter* immunity and quantitative excretion rates in Thai children. J. Infect. Dis. **168:** 754-758.

121. **Tenover, F. C., C. N. Baker, C. L. Fennell, and C. A. Ryan**. 1992. Antimicrobial resistance in *Campylobacter* species, p. 66-73. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

122. **Torres, O., and J. R. Cruz**. 1993. Protection against Campylobacter diarrhea: role of milk IgA antibodies against bacterial surface antigens. Acta Paediatr. **82:** 835-838.

123. **Trust, T. J., S. M. Logan, C. E. Gustafson, P. J. Romaniuk, N. W. Kim, V. L. Chan, M. A. Ragan, P. Guerry, and R. R. Gutell**. 1994. Phylogenetic and molecular characterization of a 23S rRNA gene positions the genus *Campylobacter* in the epsilon subdivision of the *Proteobacteria* and shows that the presence of transcribed spacers is common in *Campylobacter* spp. J. Bacteriol. **176:** 4597-4609.

124. **van Spreeuwel, J. P., G. C. Duursma, C. J. L. M. Meijer, R. Bax, P. C. M. Rosekrans, and J. Lindeman**. 1985. *Campylobacter* colitis: histological immunohistochemical and ultrastructural findings. Gut. **26:** 945-951.

125. **Walan, A., and E. Kihlstrom**. 1988. Surface charge and hydrophobicity of *Campylobacter jejuni* strains in relation to adhesion to epithelial HT-29 cells. APMIS. **96:** 1089-1096.

126. **Walker, R. I., M. B. Caldwell, E. C. Lee, P. Guerry, T. J. Trust, and G. M. Ruiz-Palacios**. 1986. Pathophysiology of *Campylobacter* enteritis. Microbiol. Rev. **50:** 81-94.

127. **Walker, R. I., D. M. Rollins, and D. H. Burr**. 1992. Studies of *Campylobacter* infection in the adult rabbit, p. 139-147. *In* Nachamkin, I., M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D. C. .

128. **Wang, Y., and D. E. Taylor**. 1990. Natural transformation in *Campylobacter* species. J. Bacteriol. **172:** 949-955.

129. **Wassenaar, T. M., N. M. C. Bleumink-Pluym, D. G. Newell, P. J. M. Nuijten, and B. A. M. van der Zeijst**. 1994. Differential flagellin expression in a *flaA flaB*$^+$ mutant of *Campylobacter jejuni*. Infect. Immun. **62:** 3901-3906.

130. **Wassenaar, T. M., N. M. C. Bleumink-Pluym, and B. A. M. van der Zeijst**. 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. EMBO J. **10:** 2055-2061.

131. **Wassenaar, T. M., B. N. Fry, and B. A. M. van der Zeijst**. 1993. Genetic manipulation of *Campylobacter*: evaluation of natural transformation and electro-transformation. Gene. **132:** 131-135.

132. **Wenman, W. M., J. Chai, T. J. Louie, C. Goudreau, H. Lior, D. G. Newell, A. D. Pearson, and D. E. Taylor**. 1985. Antigenic analysis of *Campylobacter* flagellar protein and other proteins. J. Clin. Microbiol. **21:** 108-112.

133. **Winstanley, T. G., P. S. Rice, and R. C. Spencer**. 1993. Multiple antibiotic resistance in a strain of *Campylobacter jejuni* acquired in Jordan. J. Antimicrob. Chemother. **31:** 178-179.

134. **Wooldridge, K. G., P. H. Williams, and J. M. Ketley**. 1994. Iron-responsive genetic regulation in *Campylobacter jejuni*: cloning and characterization of a *fur* homolog. J. Bacteriol. **176:** 5852-5856.

135. **Wu, Y. L., L. H. Lee, D. M. Rollins, and W. M. Ching**. 1994. Heat shock- and alkaline pH-induced proteins of *Campylobacter jejuni*: characterization and immunological properties. Infect. Immun. **62:** 4256-4260.

136. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry**. 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: Role of motility in adherence and invasion of eukaryotic cells. Mol. Microbiol. **14:** 883-893.

137. **Yao, R., and P. Guerry**. 1996. Molecular cloning and site-specific mutagenesis of a gene involved in arylsulfatase production in *Campylobacter jejuni*. J. Bacteriol. **178:** 3335-3338.

*CHAPTER 2

2.1. *CAMPYLOBACTER JEJUNI* MOTILITY AND INVASION OF CACO-2 CELLS

2.1.1. INTRODUCTION

The symptoms associated with *Campylobacter jejuni* enteritis range from Cholera-like excretory diarrhea to Shigella-like dysentery. The latter symptoms are reminiscent of an invasive infection yet there is much skepticism over whether *Campylobacter jejuni* can invade eukaryotic cells. Early work by Bukholm and Kapperud demonstrated that *C. jejuni* invaded HEp-2 and A549 cells only when co-infected with other enteropathogenic bacteria, such as *Salmonella*, *Shigella*, or *Escherichia coli* (6). de Melo *et al.* showed, however, that *Campylobacter* species could exist alone intracellularly in HEp-2 cells (7). Konkel and Joens also found that *Campylobacter* species were invasive in HEp-2 cells (15). Nonetheless, they demonstrated higher levels of HEp-2 cell invasion when *C. jejuni* was co-infected with enteroviruses (16).

The cell lines used in many of the early *Campylobacter* invasion experiments were derived from epidermoid carcinoma of the human larynx (HEp-2) and carcinoma of the human lung (A549). *Campylobacter* may not have invaded these cells efficiently because they do not accurately mimic intestinal epithelial cells. The most convincing data were obtained by Konkel *et al* (17). They infected polarized Caco-2 cells, originally derived

---

* The manuscript presented in this section is the same as that published in Infection and Immunity (1995) **63**: 4295-4300 by C. M. Szymanski, M. King, M. Haardt, and G. D. Armstrong. Dr. M. King was responsible for explaining the importance of frequency in viscosity determinations. Dr. M. Haardt was responsible for demonstrating the overlay function of the MacDraw Pro application which made the motility tracings in Figure 2.5. possible.

from a human colon adenocarcinoma. They showed that *Campylobacter* can invade Caco-2 cell monolayers to the levels found for *Salmonella* and was able to translocate across Caco-2 polarized monolayers.

The results of animal studies of *Campylobacter* enteritis suggest that invasion is a key mechanism in pathogenesis. These model studies include work with infant chickens (25, 28, 35), infant mice (23, 36), newborn piglets (3) and infant monkeys (27). Finally, Van Spreeuwel *et al.* demonstrated intracellular *C. jejuni* in colonic epithelial cells from patients with colitis and positive stool samples for *C. jejuni* (33). It is now generally accepted that *Campylobacter* can invade intestinal epithelial cells *in vivo*.

The *Campylobacter* literature clearly shows that flagella are significant virulence determinants. Yet, the role of flagella in pathogenesis is poorly understood. Some investigators believe flagella may act as adhesins while others believe flagella play a role in *Campylobacter* penetration of host epithelial cells.

To address some of the remaining deficiencies in our understanding of pathogenic mechanisms in *Campylobacter* we have taken a holistic approach to examining *C. jejuni* binding and invasion of eukaryotic cells. Our studies involved examining the effect of pH and viscosity on *Campylobacter jejuni* swimming behavior and binding and invasion of Caco-2 cells. The motivation for the studies was to establish a more reliable, *in vitro*, model system for investigating the interactions of *Campylobacter* and perhaps other enteropathogens with epithelial cells of the gastrointestinal tract.

2.1.2. MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *C. jejuni* strains E863 and ER1109 were obtained from the Provincial Laboratory of Northern Alberta. *C. jejuni* strains UA580 and UA581 were kindly provided by Dr. D. E. Taylor, University of Alberta. *Salmonella enteritidis* 710063 was kindly provided by Dr. M. Finlayson, University of Alberta.

Campylobacter organisms were stored in brain heart infusion broth (BHI, Difco Laboratories) with 10% glycerol (BDH) at -70°C. Cultures were plated on supplemented blood agar plates (Triage Microbiological Systems, Ardrossan Alberta) overnight at 37°C under microaerophilic conditions (10% CO_2, 5% O_2, 85% N_2). The organisms were then inoculated into BHI broth and incubated overnight, with constant shaking, under the same conditions. After centrifugation at 6000 rpm, the bacteria were resuspended in BHI broth and used in further experiments.

Salmonella enteritidis was maintained on BHI agar. Experiments were conducted with overnight cultures prepared in BHI broth.

Culture of Caco-2 Cells. The human epithelial cell line Caco-2 (American Type Culture Collection HTB 37) was originally derived from a human colon adenocarcinoma and is widely employed in studies of pathogen-host cell interactions due to its ability to form well-differentiated cell monolayers. The cell line is very similar to small intestinal enterocytes with respect to their structure, brush border enzymes, and time course of differentiation.

Caco-2 cells were grown at 37°C in minimal essential medium (MEM, Gibco Laboratories) supplemented with 10% (v/v) fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 and 95% air. Confluent cells were harvested by trypsinization in 0.01% ethylenediaminetetraacetic acid (EDTA, BDH) and plated in a 24-well Multiwell (Falcon) tissue culture plate. Polarized Caco-2 cell monolayers were used after 10-14 days growth similar to Konkel *et al.* (17).

Effect of pH on *Campylobacter* Motility. The effect of pH on the motility of *C. jejuni* strains UA580, E863, ER1109, and UA581 was investigated on BHI agar (0.4 % w/v) plates adjusted to pH 5.0, 7.3 and 8.5. Five µl of culture was inoculated into the center of the plates and the diameter of the resulting swarms was measured the next day.

C. jejuni UA580 was also inoculated into BHI broth, adjusted to pH 5.0, 7.3 and 8.5, and incubated overnight. The next day the organisms were negatively stained and

observed by transmission electron microscopy. The pH of the broth was also checked to ensure that there was no change. The viability of the organisms was determined by plating serial dilutions at 5 hour intervals throughout the incubation period.

Invasion and Binding Assays. Caco-2 cell monolayers in MEM/FBS or MEM/FBS and carboxymethylcellulose (CMC, Sigma) were infected with approximately 10^9 *C. jejuni* organisms for 5 hours at 37°C in a humidified atmosphere. Caco-2 cell monolayers were infected with *S. enteriditis* for 2 hours. The infected cells were then washed and incubated with 100 μg/ml gentamicin (Gibco BRL) in MEM/FBS at 37°C for 1 hour to kill any extracellular organisms. The cells were washed again and treated with 1% Triton X-100 (Sigma) in PBS at 37°C for 5 min to release intracellular organisms. Dilutions from each well were plated on BHI agar. We have previously determined that the Triton X-100 treatment does not affect the viability of *C. jejuni*.

The total number of organisms bound was determined simultaneously by performing the invasion assay excluding gentamicin treatment. Since the modified version of the assay yielded the number of organisms bound plus internalized, the difference between total bound and internalized was taken to give the number of bound organisms.

Viscosity Measurements. Viscosity measurements were done using a Cannon-Fenske Routine Type Viscometer No. 475. All measurements were done at 37°C. The kinematic viscosity of the sample, in centistokes, was obtained by multiplying the efflux time, in seconds, by the viscometer constant at 37°C. To determine the viscosity in centipoise, the kinematic viscosity was multiplied by the density of the solutions in grams per milliliter.

Bacterial Vibrational Frequency and Velocity Measurements. *Campylobacter jejuni* UA580, E863, and ER1109 were grown in BHI broth in a microaerobic environment, overnight, at 37°C, with constant shaking. *E. coli* and *S. enteriditis* standing cultures were grown in BHI broth, overnight, at 37°C.

The next day, the organisms were added to a 24-well tissue culture plate containing MEM/FBS alone or MEM/FBS with 0.6% CMC (w/v) and incubated for 5 hours at 37°C in

a humidified atmosphere. A small drop of each culture was placed under a sealed cover slip on a microscope slide. Video recordings of the bacteria were obtained using a Carl Zeiss microscope, JVC CCD camera, and Sony SL-2500 video cassette recorder. When the videotape was played back on the monitor, the tape speed was manually slowed to allow measurement of bacterial vibrational frequencies and traveling velocities.

Tracing Bacterial Movements. Photographs of the videotape were taken with an Appligene High Performance CCD Imaging System at intervals of 0.2 second. The images were saved and analyzed as TIFF files.

2.1.3. RESULTS

Effect of pH on *Campylobacter* Motility. The control strain (UA581) used in the motility experiments was a fla^-, mot^- *C. jejuni*. We included this strain to monitor non-propulsive bacterial motion on motility agar. The other strains were fla^+, mot^+. Strain UA580 was a lab strain passaged multiple times since its isolation. The other strains were recent clinical isolates. We found that lowering the pH from 7.3 to 5.0 decreased the ability of all three fla^+, mot^+ *C. jejuni* strains to swarm on motility agar (Figure 2.1). Electron microscopy observations revealed that the morphology of UA580 flagellae were unaltered at pH 5.0 (Figure 2.2). Moreover, the viability of the *C. jejuni* strains did not decrease at the lower pH levels. Increasing the pH to 8.5 had little effect on *C. jejuni* motility.

Binding and invasion in viscous solutions. We speculated that although rapidly swimming *C. jejuni* may make frequent contact with the Caco-2 cells, the rapid movement of the organisms may prevent them from becoming firmly attached to the surface of these cells. If the organisms were moving more slowly, *C. jejuni* may make less frequent contacts with the Caco-2 cells but more of these collisions may result in irreversible binding and internalization.

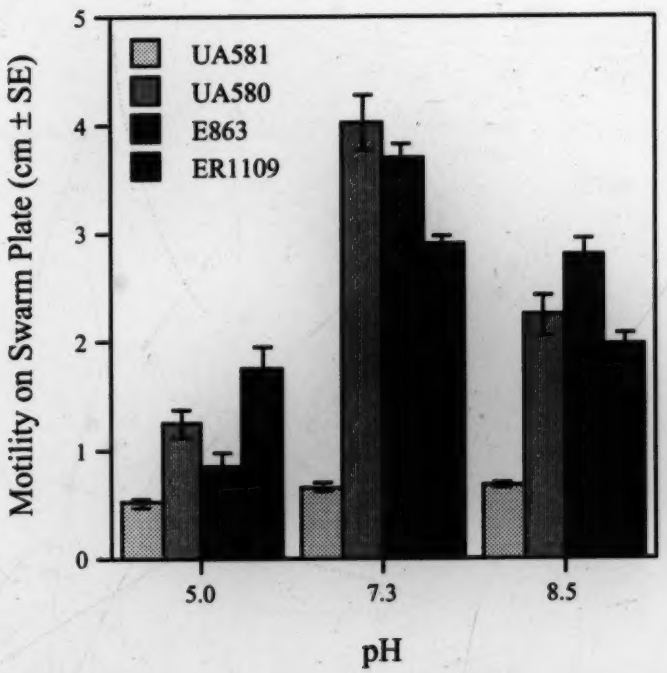
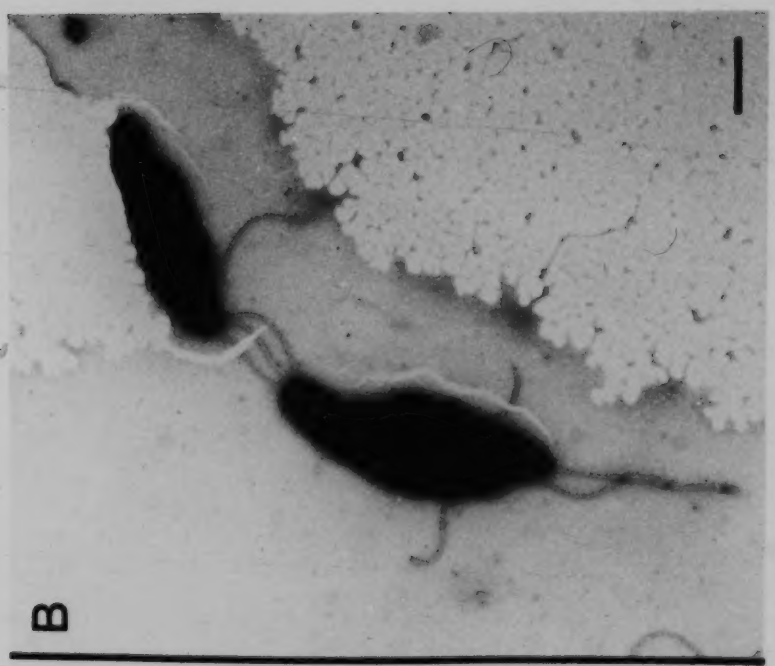


FIGURE 2.1. *Campylobacter* motility on swarm plates at various pH values. The results shown for *C. jejuni* UA581 were determined from quadruplicate experiments. The results shown for *C. jejuni* UA580 and E863 were determined from triplicate experiments while results for *C. jejuni* ER1109 represent a single experiment. Inocula: UA581: $1.0 \pm 0.06 \times 10^7$; UA580: $1.1 \pm 0.25 \times 10^7$; E863: $0.75 \pm 0.16 \times 10^7$; ER1109: $0.75 \pm 0.10 \times 10^7$.

FIGURE 2.2. Electron micrographs illustrating the morphology of overnight cultures of *C. jejuni* UA580 grown at (A) pH 5.0 and (B) pH 7.3. Note that the flagellae remain intact without any observable deformations. The solid bar represents 1 μm.


B

A

To test this hypothesis we modified the standard tissue culture invasion assay so it more closely resembled the conditions in the human intestines. We specifically wanted to reproduce the physical characteristics of the mucoid secretions covering intestinal epithelial cells. We chose to use carboxymethylcellulose (CMC) since this is more chemically defined than purified mucin, is non-toxic to tissue culture cells and approximates the viscosity of intestinal mucus.

As predicted by our hypothesis, *C. jejuni* invasion increased when we used CMC to mimic the viscosity of intestinal mucus on the Caco-2 cells (Figure 2.3). However, the binding of *C. jejuni* also increased (Figure 2.4). We also examined *Salmonella enteritidis* in the conventional and modified Caco-2 invasion assay. In contrast to *C. jejuni*, binding and invasion of *S. enteritidis* did not change in CMC-supplemented medium (Figure 2.4).

Effect of viscosity on motility. To examine the relationship between viscosity and the swimming behavior of the microorganisms we used a videotaping system to record the movements of *C. jejuni* and *S. enteriditis* in regular and high viscosity culture medium. In regular tissue culture medium all the bacteria displayed typical smooth swimming behavior (runs) punctuated by periods of tumbling (Figure 2.5). However, *S. enteriditis* traveled more slowly for shorter distances during periods of smooth swimming than any of the *C. jejuni* isolates.

In viscous solutions, *C. jejuni* and *S. enteriditis* showed smooth swimming patterns punctuated by pauses in motion rather than tumbling behavior. At higher viscosities, *C. jejuni* exhibited a darting motility. During periods of smooth swimming *C. jejuni* traveled much farther with greater velocity in viscous medium (Figure 2.6). In contrast, the distance traveled and velocity of *S. enteriditis* was significantly lower during periods of smooth swimming in viscous solutions.

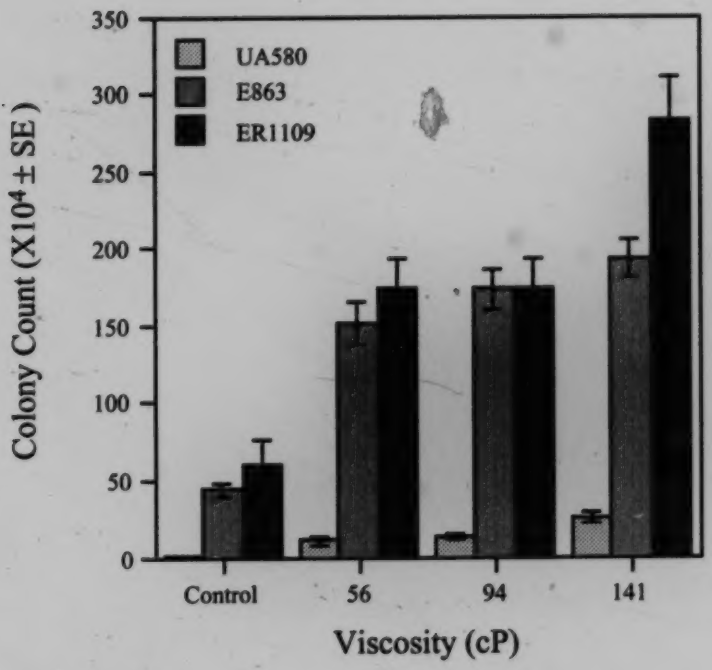


FIGURE 2.3. Concentration-dependent increase in *C. jejuni* invasion of Caco-2 cells in CMC-supplemented MEM solutions. Control represents *C. jejuni* invasion of Caco-2 cells in MEM alone (conventional invasion assay). Mean CFU per well were determined from duplicate sets of wells per experiment. Each experiment was done in duplicate. All increases in invasion were determined to be statistically significant by the t-test ($p < 0.05$).

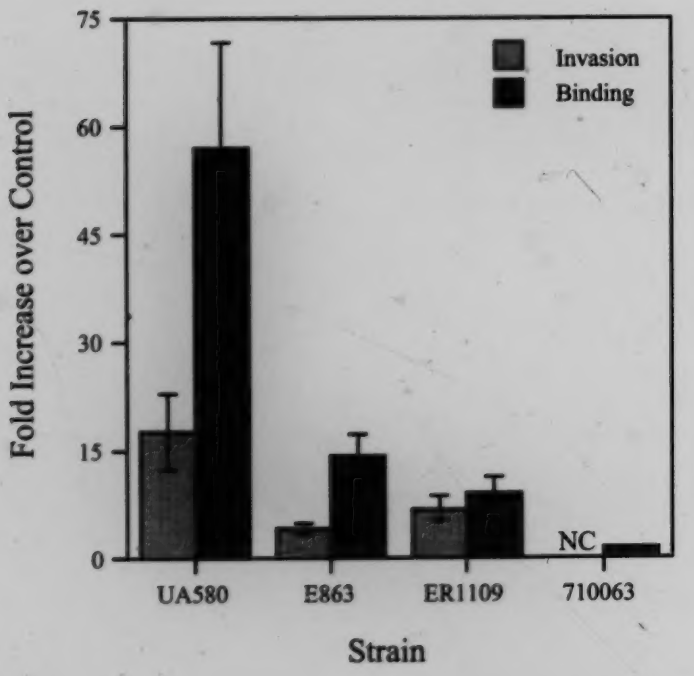


FIGURE 2.4. Comparison between binding and invasion in 141 cP (0.6% w/v) CMC solutions. *S. enteriditis* 710063 is also shown. Mean CFU per well were determined from duplicate (invasion) or triplicate (binding) sets of wells per experiment. Each experiment was done in duplicate or triplicate. The fold increase over the control was determined ± SE. Control values for binding and invasion, respectively, are as follows: UA580 56.0 ± 7.2 x 10^5 and 14.7 ± 2.3 x 10^4; E863 14.8 ± 2.7 x 10^7 and 27.1 ± 3.5 x 10^5; ER1109 55.2 ± 12.9 x 10^6 and 38.9 ± 4.7 x 10^5; 710063 13.2 ± 1.4 x 10^6 and 11.1 ± 1.8 x 10^4. NC = no change. All changes in *Campylobacter* binding and invasion were determined to be statistically significant by the t-test ($p < 0.05$).

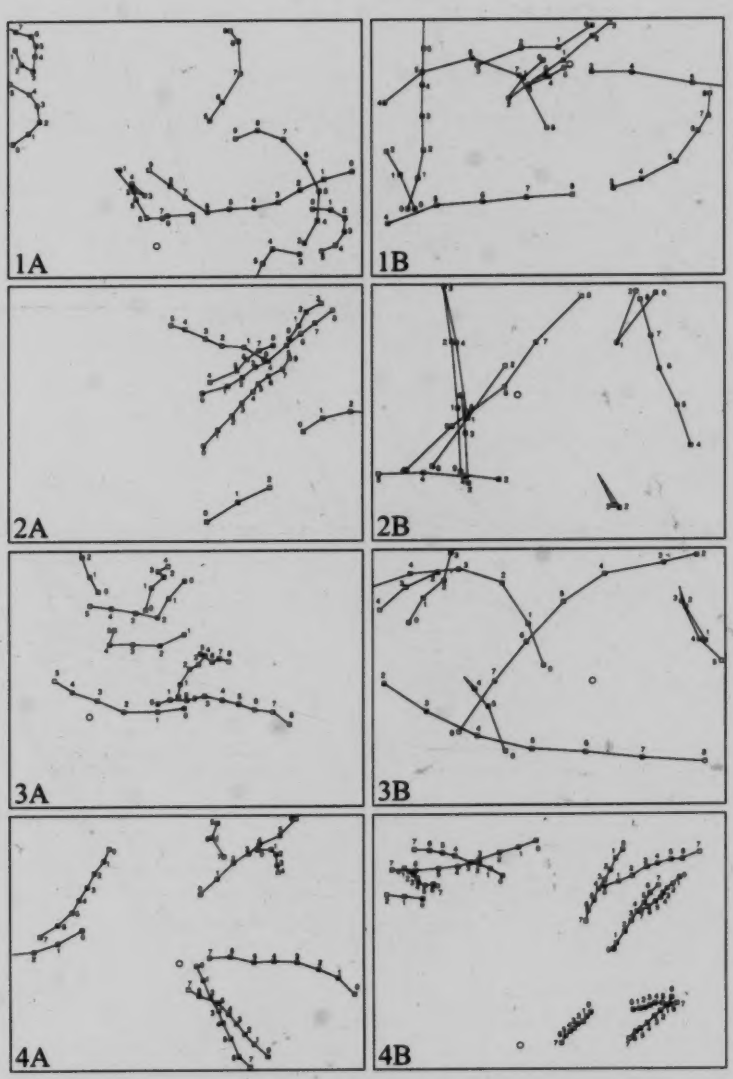


FIGURE 2.5. Movements of *C. jejuni* (1-3) and *S. enteriditis* (4) in (A) regular medium and (B) viscous medium (0.6% CMC, w/v). Photographs of the videotape were taken at intervals of 0.2 second and the bacterial positions, at each interval, are designated by open squares. The circle represents the reference point on the television screen.

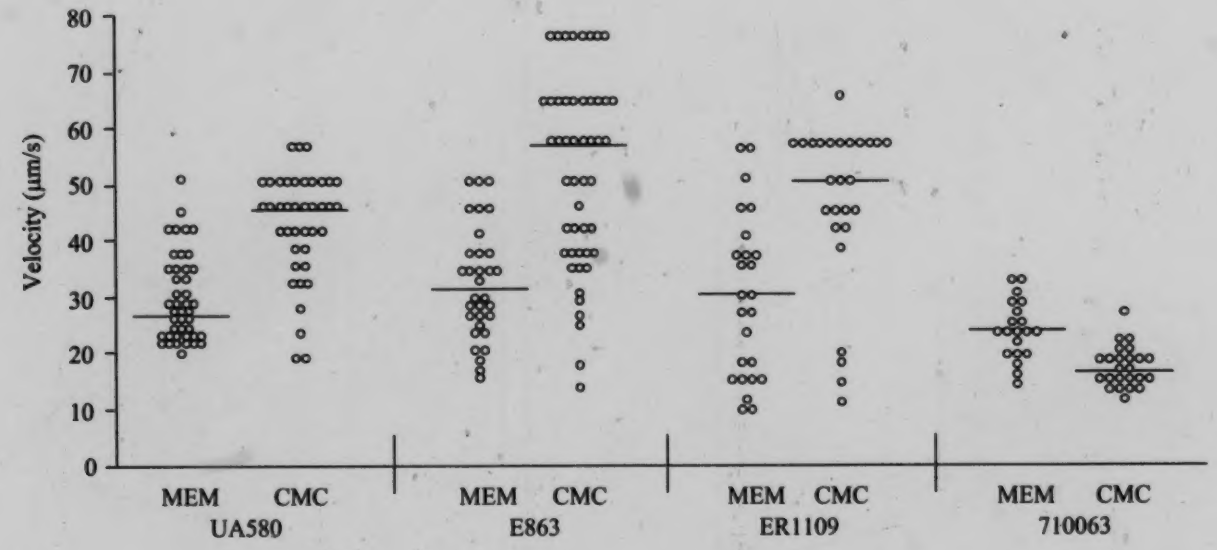


FIGURE 2.6. Velocity of *C. jejuni* UA580, E863 and ER1109 and *S. enteriditis* 710063 in regular tissue culture medium (MEM) and viscous medium (0.6% CMC, w/v). The median of each group of data points is shown.

2.1.4. DISCUSSION

Motility should play an important role for *Campylobacter* colonization since the organism has to penetrate mucus in order to adhere to and invade surface epithelial cells. *C. jejuni* is motile by means of one or two polar flagella. The organism contains two copies of the flagellin gene. The expression of these genes is controlled by two unique promoters, at least one of which responds to environmental signals (2). The evolution of this complex flagellar expression system in *C. jejuni* emphasizes the importance of motility to its survival.

Increases in viscosity resulting in increased bacterial motility have previously been described as a general behavioral phenomenon in bacteria (12, 29, 31). However, the low viscosity range at which these observations were made is unrepresentative of the viscosity of mucoid secretions found in the gastrointestinal tract. Ferrero and Lee compared *C. jejuni* motility with conventional rod-shaped bacteria in a viscous environment (9). Although they observed that *Campylobacter* was motile in viscous, mucoid-like solutions that immobilized other bacteria, they did not extend their investigations to examining the effect of viscosity on infectivity (9). Other groups described the effect of mucus or mucin, on the pathogenesis of *C. jejuni,* but these investigations were directed at determining whether mucus components mediated bacterial attachment to epithelial cells (8, 21, 22).

In our experiments we examined the physical effect of viscosity on attachment and invasion of *C. jejuni* and *S. enteritidis* in Caco-2 cells. However, in determining the impact of viscosity on the behavior of microorganisms it is necessary to account for the effect of motility on the viscosity of the solution through which the bacteria are traveling. As microorganisms swim they vibrate with a characteristic frequency that applies a strain to the viscous solution. With any gel-like material, the apparent viscosity varies with the strain (14). In practical terms, this means that different microorganisms, vibrating at different frequencies may actually behave as if they were in solutions of quite different

viscosity. The corollary to this is that microorganisms modify the viscosity of a solution as they swim through it.

The vibrational frequency of the organisms used in our experiments was determined from the videotape records to be approximately 20-30 Hz. In experiments by Mantle *et al.*, Sellers *et al.*, and Bell and Allen, the viscosity of reconstituted intestinal mucus ranged between 178-316 cP at 16 Hz and could be estimated to be approximately 140 cP at 30 Hz, based on the typical frequency dependence in this range (4, 20, 30). Therefore, although we cannot be certain of the actual viscosity experienced by the *C. jejuni* and *S. enteriditis*, this should be similar for each of them. As a control, we also measured the vibrational frequency of *E. coli* (5-10 Hz) which we found to be similar to the frequency of *E. coli* (8-13 Hz) determined by Berg and Turner (5).

In the presence of viscous solutions, the invasion of *C. jejuni* increases as a result of increased attachment. We believe that increases in attachment may be due to more contacts or, perhaps, impact between *Campylobacter* and the host cell surface. Berg and Turner showed that viscous solutions form a highly structured network which can be easily penetrated by microscopic organisms (5). From our videotaping observations, *C. jejuni* can easily maneuver through the gel matrix of a 0.6% (w/v) CMC solution. The velocity of *C. jejuni* in 0.6% CMC was significantly greater than its velocity in MEM alone. In contrast the velocity of *S. enteritidis* significantly decreased in 0.6% CMC/MEM.

Lee *et al.* examined scrapings of mucosa from cecal tissue of infected mice (19). They also found that *C. jejuni* was highly motile in the tissue. Interestingly, the organisms moved extremely rapidly across the field of view in parallel streams. The bacteria seemed to track along the mucus strands. Ferrero and Lee measured the pathlengths of *C. jejuni* in two solutions of different viscosities (9). They found an abrupt increase in the proportion of cells that displayed longer pathlengths during smooth swimming in a high viscosity medium. It is obvious from our tracings that all three strains of *C. jejuni* also showed longer swimming pathlengths in viscous solutions. In contrast, movement of *S. enteriditis*

was reduced in solutions of similar viscosity, as has been previously described (9). We suggest that the increased directionality of *Campylobacter* motility in viscous solutions may favor an increase in the frequency of collisions with host epithelial cells. This phenomenon would increase the number of contacts between the organisms and the host cells and therefore increase binding.

It was previously believed that *Campylobacter* flagella acted as adhesins. Then, Wassenaar *et al.* found that antibodies directed against the flagella did not inhibit attachment to INT-407 cells (34). McSweegan and Walker found that sheared flagella were not effective in blocking the attachment of whole *C. jejuni* cells to INT-407 cells (22). Later, several laboratories demonstrated that flagella and/or motility is required for the internalization of *C. jejuni in vitro* and *in vivo* (1, 26, 34, 36, 37).

Motility of an organism can be broken down into two components: translational motion, represented by velocity, and vibrational motion, represented by frequency. Flagella rotate about their long axes with a measurable frequency (13). We speculate that *Campylobacter* may have adopted a unique form of motility that allows the organisms to function efficiently in a viscous medium such as mucus. Greenberg and Canale-Parola suggested that certain motile bacteria were able to swim through viscous environments because they may posess a specialized motility apparatus (12). Ferrero and Lee also described two mechanisms of motility for *C. jejuni* (9). At low viscosities they observed that *Campylobacter* behaved much like other flagellated bacteria. The organisms relied primarily on their flagellae for propulsion. At higher viscosities Ferrero and Lee speculated that *C. jejuni* was able to overcome the problem of "flagellar dampening" caused by viscous media by developing a different mechanism of motility. While other organisms have a low minimum inhibitory viscosity, *Campylobacter* remains motile in highly viscous solutions (i.e. > 100 cP) (9). Also, *Campylobacter* moves much faster than other intestinal bacteria.

The importance of motility to *C.jejuni* has already been emphasized. The organism contains two copies of the flagellin gene, at least one of which is independently and environmentally regulated. The organism has repeatedly been shown to be well adapted to movement in viscous environments. Our results also showed that although the flagellae remain intact without any observable morphological changes, the organisms lose motility but remain viable at pH 5. Thus, even if the organisms remain viable in an acidic environment like the stomach *C.jejuni* may not be able to initiate an infection in this environment. It has been shown that expression of *flaB* is reduced when the organisms are grown at pH 5 at 42°C (2). However, when *C. jejuni* was grown at 37°C, *flaB* expression slightly increased (2). Since our experiments were done at 37°C, it is unlikely that changes in *flaB* expression contributed to the reduced motility of *C. jejuni* at pH 5.

Our results suggest that the motility of *C. jejuni* may play several key roles in pathogenesis: (1) involvement in tissue tropism, (2) transport through mucus toward surface epithelial cells, and (3) increases the efficiency of attachment and invasion of host cells. Experiments are currently underway to further elucidate the role of *C. jejuni* motility in pathogenesis.

2.2. RELATED WORK

This section describes the importance of frequency when determining the viscosity of a solution and the procedure used to determine the appropriate viscosities for this study. A representative figure demonstrating the actual bacterial movements used to form a tracing is also shown. Finally, results from experiments which investigate *C. jejuni* binding and invasion in solutions of lower viscosity are also described.

We examined the physical property, viscosity, and its effect on the attachment and invasion of *C. jejuni* and *S. enteriditis*. In our experiments we considered the liquid-like character of viscous solutions (G", viscous modulus) which is examined by applying a shear stress (in our experiments, gravity) and measuring the resulting strain (flow). The liquid-like character is quantified by the viscosity, the ratio of the stress to the rate of strain. With any gel-like material, the apparent or measured viscosity varies greatly with the applied stress (or with the resultant strain rate) (14). If one plots the dependence of apparent viscosity on strain rate, the curve exhibits several orders of magnitude difference between the low and high strain rate limits of viscosity.

In experiments by Mantle *et al.*, the viscosity of reconstituted human intestinal mucus has been measured to be 250 cP at 16 Hz (20). From their observations, G" showed a certain amount of frequency dependence. These results are similar to those observed in studies on the rheological properties of pig intestinal mucus and mucin gels (4, 30). It has also been shown that pig gastric, intestinal and colonic mucus gels all have very similar rheological properties as fresh mucus(30). The vibrational frequency of the organisms used in our viscosity experiments was determined to be 20-30 Hz. Since, the rate of strain in our experiments is higher, the apparent viscosity should be lower. We mentioned earlier that the viscosity of intestinal mucus could be estimated to be approximately 140 cP at 30 Hz. Figure 2.7 is a graph of mucus viscosity versus strain rate. The points at 0.159 Hz and 1.59 Hz were determined from results reported by Sellers *et al.* (30). The point at 15.9 Hz is an average of the viscosities reported by Sellers *et al.*,

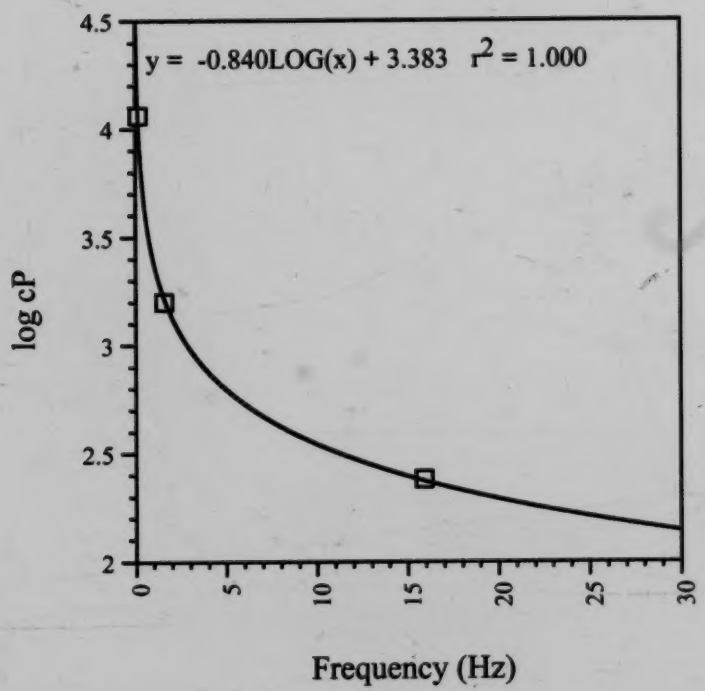


Figure 2.7. Viscosity of mucus versus strain rate. The viscosity at 15.92 Hz is the mean of the viscosities reported by Sellers *et al.*, Mantle *et al.*, and Bell and Allen (see text).

Mahtle *et al.*, and Bell and Allen (4, 20, 30). According to Figure 2.7, the viscosity at 30 Hz was extrapolated to be 140 cP which is the same as the viscosity of the 0.6% CMC solution.

Bacterial movements were traced by comparing photographs taken at 0.2 second in the videotape. As previously mentioned, the images were saved and analyzed as TIFF files. Figure 2.8 represents one such file with *C. jejuni* UA580 in viscous solution. The final tracing is shown in the top left corner of the figure. In order to facilitate tracing for the reader, four bacteria were selected with different symbols and their location shown in each frame.

Effect of low viscosity on *C. jejuni* binding and invasion. We previously showed that binding and invasion increases for *C. jejuni* strains UA580, E863, and ER1109 in highly viscous solutions, 55-150 cP using CMC. However, we also investigated changes in invasion and binding at low viscosities, 1-4 cP, using herring sperm DNA, gelatin, and Percoll. All of the results are summarized in Table 2.1.

In 1960, Shoesmith reported that slight increases in viscosity (1-3 cP) increased bacterial motility (31). Further increases in viscosity decreased motilty. He concluded that motility is affected by changes in viscosity. Schneider and Doetsch confirmed and extended Shoesmith's observations (29). They examined a diverse group of flagellated bacteria: gram positive and gram negative, aerobic, facultative, and anaerobic, monotrichous and peritrichous, large and small, sporeformers and nonsporeformers, rods and cocci. Schneider and Doetsch showed that Shoesmith's observations were a general behavioral phenomenon of motile bacteria. In 1977, Greenberg and Canale-Parola examined immobilizing viscosity ranges (12). Most strains examined lost translational motility as well as vibrational motility at a relatively low viscosity. They concluded that bacteria that are able to swim through viscous natural environments may have a selective and ecological advantage over bacteria without this ability. Then, in 1988, Ferrero and Lee compared the motility of *Campylobacter jejuni* with conventional rod-shaped bacteria in a

FIGURE 2.8. Television screen images of *C. jejuni* UA580 movement in viscous solution. Bacterial movements were traced by comparing photographs taken at 0.2 second in the videotape. Four bacteria were selected with different symbols (closed circle, asterisk, closed triangle, and closed diamond) and their location shown in each frame. The final tracing (as seen in Fig. 2.5) is shown in the top left corner of this figure. The "X" on the final tracing represents the reference point on the television screen.

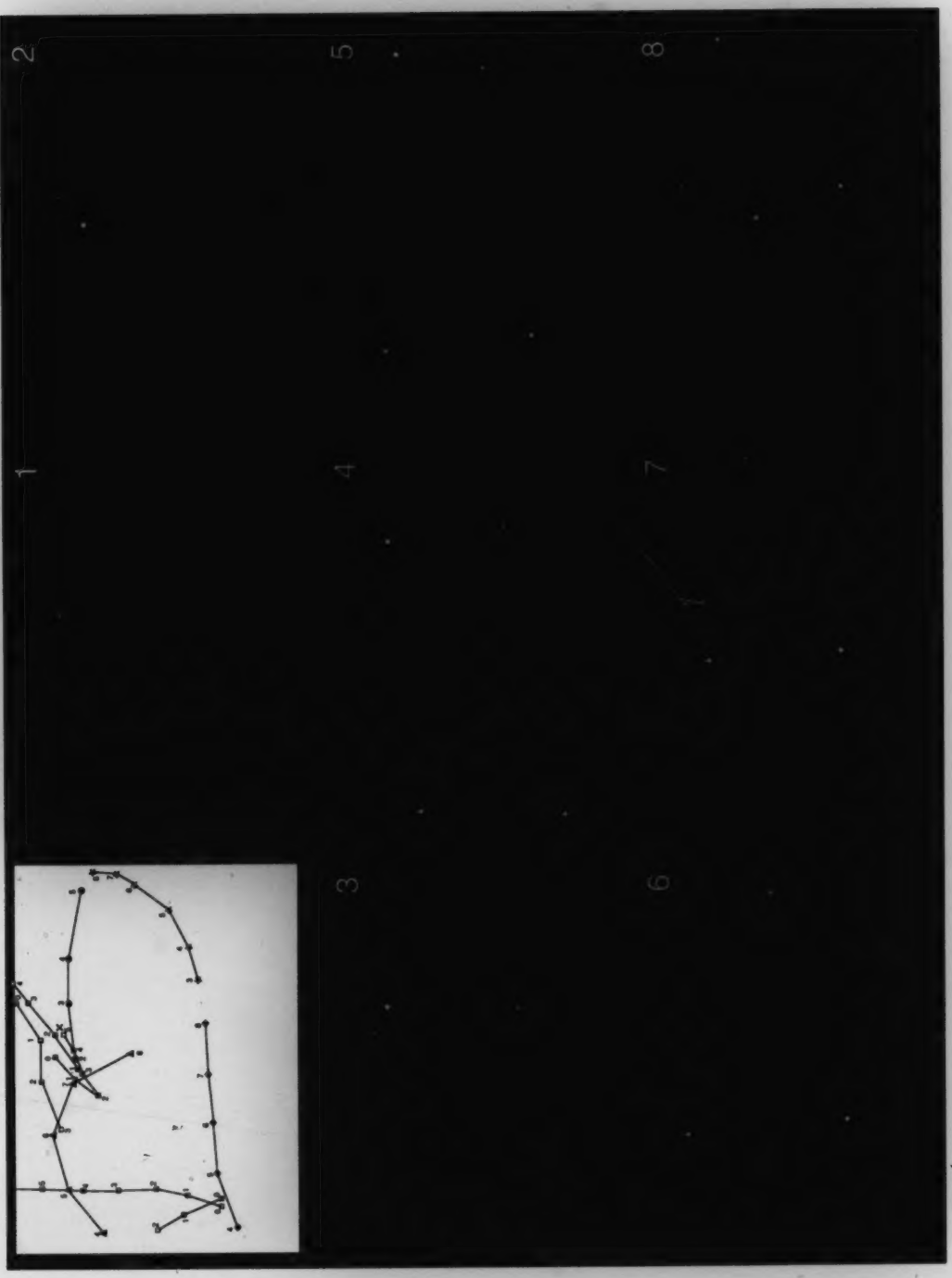

2
5
8
1
4
7
3
6

TABLE 2.1. Summary of the effect of viscosity on *C. jejuni* binding and invasion.

Viscosity	Reagent	Conditions	UA580	E863	ER1109
Low viscosity	Gelatin	Binding	No change	No change	No change
(1-4 cP)		Invasion	Increase	Increase	Increase
	Sperm DNA	Binding	No change	No change	Increase
		Invasion	Increase	Increase	Increase
	Percoll	Binding	No change	No change	No change
		Invasion	Increase	Increase	Increase
High viscosity	CMC	Binding	Increase	Increase	Increase
(55-150 cP)		Invasion	Increase	Increase	Increase

viscous environment (9). Again, they observed an initial increase in swimming velocity in solutions of low viscosity for all bacteria tested. But, they observed that only Campylobacters were actively motile in highly viscous solutions.

It has been shown that all bacteria, including *Campylobacter jejuni*, show an increase in their swimming velocity at viscosities slightly greater than water (1-4 cP). In our studies of the low viscosity range, all three *Campylobacter* isolates exhibited a significant increase in invasion. However, unlike results in the high viscosity range, attachment to CaCo-2 cells was not changed. The results imply that the motility of the organism may be involved in the invasive process. Several laboratories have demonstrated that flagella and/or motility is required for the internalization of *C. jejuni in vitro* and *in vivo* (11, 26, 34, 36). We tested this by determining the effect of low viscosity solutions on the invasion of non-motile *C. jejuni* UA581. *C. jejuni* UA581 demonstrated a decrease in invasion of Caco-2 cells in all three of the reagents examined. These results support the idea that motility is important for invasion.

We can only speculate on the significance motility has on invasion. Perhaps the impact to the cell upon collision induces the uptake of the organism. Tomita and Kangasaki also speculated that the physical impact caused by bacterial motility or by centrifugation enhanced phagocytosis of bacteria by macrophages (32). Alternatively, the frequency of the bacterial vibration may act as a signal to the cytoskeletal network signalling its uptake. Although the second theory may seem somewhat obscure, it has been shown that microtubule networks actively transmit vibrational signals (10, 18). Unlike most invasive organisms, we (Chapter 6) as well as Oelschlaeger *et al.* have shown that Campylobacters require microtubules as well as microfilaments for invasion (24).

The viscosity range encountered in this system may not be encountered *in vivo*, although it is possible that similar conditions exist when the organism is ingested with raw milk. Even if these conditions are not found *in vivo*, the observation is interesting because the results demonstrate that *C. jejuni* is capable of sensing the viscosity of its surroundings

resulting in changes in swimming patterns and infectivity. Also, since *Campylobacter* invasion is uncoupled from binding, this system may be useful in providing further information on the invasive mechanisms of *Campylobacter in vivo*.

2.3. REFERENCES

1. **Alm, R. A., P. Guerry, and T. J. Trust**. 1993b. Distribution and polymorphism of the flagellin genes from isolates of *Campylobacter coli* and *Campylobacter jejuni*. J. Bacteriol. **175:** 3051-3057.

2. **Alm, R. A., P. Guerry, and T. J. Trust**. 1993a. The *Campylobacter* σ54 *flaB* flagellin promoter is subject to environmental regulation. J. Bacteriol. **175:** 4448-4455.

3. **Babakhani, F. K., G. A. Bradley, and L. A. Joens**. 1993. Newborn piglet model for Campylobacteriosis. Infect. Immun. **61:** 3466-3475.

4. **Bell, A. E., and A. Allen**. 1984. Functional interactions of gastric mucus glycoprotein. Int. J. Biol. Macromol. **6:** 309-315.

5. **Berg, H. C., and L. Turner**. 1979. Movement of microorganisms in viscous environments. Nature. **278:** 349-351.

6. **Bukholm, G., and G. Kapperud**. 1987. Expression of *Campylobacter jejuni* invasiveness in cell cultures coinfected with other bacteria. Infect. Immun. **55:** 2816-2821.

7. **deMelo, M. A., G. Gabbiani, and J. Pechere**. 1989. Cellular events and intracellular survival of *Campylobacter jejuni* during infection of HEp-2 cells. Infect. Immun. **57:** 2214-2222.

8. **deMelo, M. A., and J. C. Pechere**. 1988. Effect of mucin on *Campylobacter jejuni* association and invasion on HEp-2 cells. Microbial Pathog. **5:** 71-76.

9. **Ferrero, R. L., and A. Lee**. 1988. Motility of *Campylobacter jejuni* in a viscous environment: comparison with conventional rod-shaped bacteria. J. Gen. Microbiol. **134:** 53-59.

10. **Freedman, D.** 1994. Quantum consiousness. Discover. 89-98.

11. **Grant, C. C. R., M. E. Konkel, J. W. Cieplak, and L. S. Tompkins**. 1993. Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. Infect. Immun. **6:** 1764-1771.

12. **Greenberg, E. P., and E. Canale-Parola**. 1977. Motility of flagellated bacteria in viscous environments. J. Bacteriol. **132:** 356-358.

13. **Holwill, M. E. J., and R. E. Burge**. 1963. A hydrodynamic study of the motility of flagellated bacteria. Arch. Biochem. Biophys. **101:** 249-260.

14. **King, M.** 1980. Viscoelastic properties of airway mucus. Federation Proc. **39:** 3080-3085.

15. **Konkel, M. E., and L. A. Joens**. 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect. Immun. **57:** 2984-2990.

16. **Konkel, M. E., and L. A. Joens**. 1990. Effect of enteroviruses on adherence to and invasion of HEp-2 cells by *Campylobacter* isolates. Infect. Immun. **58:** 1101-1105.

17. **Konkel, M. E., D. J. Mead, S. F. Hayes, and W. Cieplak Jr.** 1992b. Translocation of *Campylobacter jejuni* across human polarized epithelial cell monolayer cultures. J. Infect. Dis. **166:** 308-315.

18. **Lahoz-Beltra, R., S. R. Hameroff, and J. E. Dayhoff**. 1993. Cytoskeletal logic: a model for molecular computation via Boolean operations in microtubules and microtubule-associated proteins. BioSystems. **29:** 1-23.

19. **Lee, A., J. L. O'Rourke, P. J. Barrington, and T. J. Trust**. 1986. Mucus colonization as a determinant of pathogenicity in intestinal infection by *Campylobacter jejuni*: a cecal mouse model. Infect. Immun. **51:** 536-546.

20. **Mantle, M., G. Stewart, G. Zayas, and M. King**. 1990. The disulphide-bond content and rheological properties of intestinal mucins from normal subjects and patients with cystic fibrosis. Biochem. J. **266:** 597-604.

21. **McSweegan, E., D. H. Burr, and R. I. Walker**. 1987. Intestinal mucus gel and secretory antibody are barriers to *Campylobacter jejuni* adherence to INT 407 cells. Infect. Immun. **55:** 1431-1435.

22. **McSweegan, E., and R. I. Walker**. 1986. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect. Immun. **53:** 141-148.

23. **Newell, D. G., and A. Pearson**. 1984. The invasion of epithelial cell lines and the intestinal epithelium of infant mice by *Campylobacter jejuni/coli*. J. Diarrhoeal Dis. Res. **2:** 19-26.

24. **Oelschlaeger, T. A., P. Guerry, and D. J. Kopecko**. 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. Proc. Natl. Acad. Sci. **90:** 6884-6888.

25. **Ruiz-Palacios, G. M., E. Escamilla, and N. Torres**. 1981. Experimental *Campylobacter* diarrhea in chickens. Infect. Immun. **34:** 250-255.

26. **Russell, R. G., and J. D. C. Blake**. 1994. Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. Infect. Immun. **62:** 3773-3779.

27. **Russell, R. G., M. O'Donnoghue, J. D. C. Blake, J. Zulty, and L. J. DeTolla**. 1993. Early colonic damage and invasion of *Campylobacter jejuni* in experimentally challenged infant *Macaca mulatta*. J. Infect. Dis. **168:** 210-215.

28. **Sanyal, S. C., K. M. Islam, P. K. Neogy, M. Islam, P. Speelman, and M. I. Huq**. 1984. *Campylobacter jejuni* diarrhea model in infant chickens. Infect. Immun. **43:** 931-936.

29. **Schneider, W. R., and R. N. Doetsch**. 1974. Effect of viscosity on bacterial motility. J. Bacteriol. **117:** 696-701.

30. **Sellers, L. A., A. Allen, E. Morris, and S. R. Murphy**. 1983. Rheological studies on pig gastrointestinal mucous secretions. Biochem. Soc. Trans. **11:** 763-764.

31. **Shoesmith, J. G.** 1960. The measurement of bacterial motility. J. Gen. Microbiol. **22:** 528-535.

32. **Tomita, T., and S. Kanegasaki**. 1982. Enhanced phagocytic response of macrophages to bacteria by physical impact caused by bacterial motility or centrifugation. Infect. Immun. **38:** 865-870.

33. **van Spreeuwel, J. P., G. C. Duursma, C. J. L. M. Meijer, R. Bax, P. C. M. Rosekrans, and J. Lindeman**. 1985. *Campylobacter* colitis: histological immunohistochemical and ultrastructural findings. Gut. **26:** 945-951.

34. **Wassenaar, T. M., N. M. C. Bleumink-Pluym, and B. A. M. van der Zeijst**. 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. EMBO J. **10:** 2055-2061.

35. **Welkos, S. L.** 1984. Experimental gastroenteritis in newly-hatched chicks infected with *Campylobacter jejuni*. J. Med. Micro. **18:** 233-248.

36. **Yanagawa, Y., M. Takahashi, and T. Itoh**. 1994. [The role of flagella of *Campylobacter jejuni* colonization in the intestinal tract in mice and the cultured-cell infectivity]. Japanese J. Bacteriol. **49:** 395-403.

37. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry**. 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: Role of motility in adherence and invasion of eukaryotic cells. Mol. Microbiol. **14:** 883-893.

*CHAPTER 3

3.1. INTERACTIONS BETWEEN *CAMPYLOBACTER JEJUNI* AND LIPIDS

3.1.1. INTRODUCTION

Campylobacter jejuni is a common cause of gastroenteritis in developed and developing countries. Despite the prevalence of *C. jejuni* infections very little is known about the interactions of these organisms with intestinal cells. In particular, the nature of the host cell receptor remains undefined. McSweegan and Walker as well as Moser *et al.* investigated the role of carbohydrates in the adherence of *Campylobacter* to epithelial cells (31, 33). Both groups found that fucose and mannose were inhibitory, but neither sugar completely inhibited adhesion. Cinco *et al.* also demonstrated that fucose was partially inhibitory (8). Meanwhile, Næss *et al.* demonstrated a concentration independent inhibitory affect with rhamnose (34). However, other groups could not demonstrate a role for carbohydrates in the attachment of *Campylobacter* to epithelial cells (10, 24, 32).

We previously showed that motility plays several key roles in *C. jejuni* pathogenesis, including tissue tropism, penetration of mucus lining the intestinal tract, and increasing the efficiency of *C. jejuni* attachment to host epithelial cells (46). To further study attachment we wanted to determine whether certain carbohydrate structures were involved in *C. jejuni* attachment to host cells. To investigate this possibility, we compared *C. jejuni* attachment to Chinese hamster ovary (CHO) cells and to CHO cell mutants with defined defects in complex carbohydrate biosynthesis (42). We also attempted to inhibit the binding of *C. jejuni* to CHO cells with simple sugars.

---

* The manuscript presented in this section is the same as that published in Infection and Immunity (1996) **64**: 3467-3474 by C. M. Szymanski and G. D. Armstrong.

In 1989, Lingwood *et al.* identified a gastric lipid, later shown to be phosphatidylethanolamine, as a receptor for *Helicobacter pylori* (27, 28). Lipid binding has also been described for several other organisms such as *Helicobacter mustelae* (13, 14), *Neisseria gonorrhoeae* (9, 44), *Chlamydia trachomatis* and *Chlamydia pneumoniae* (22), *Mycoplasma pneumoniae* (23, 38), *Bordetella pertussis* (4), *Borrelia burgdorferi* (1), *Escherichia coli* (25), and *Pseudomonas aeruginosa* (26). Since *Campylobacter* and *Helicobacter* are so closely related (17, 35), we wanted to determine whether *C. jejuni* also binds to lipids. This paper describes these findings.

3.1.2. MATERIALS AND METHODS

The following simple sugars were used in the inhibition studies: mannose, fucose and N-acetylglucosamine (GlcNAc) were purchased from Sigma; glucose, maltose and galactose were purchased from Fisher Scientific.

Cholesterol (Ch), phosphatidylinositol (PI) from bovine liver, phosphatidylglycerol (PG) from egg yolk, synthetic lysophosphatidylethanolamine (LysoPE), cardiolipin (Ca) from bovine heart, sphingomyelin (SP), gangliosides (Ga), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), all from bovine brain, were also purchased from Sigma. Lysophosphatidylcholine (LysoPC), glycerylphosphorylcholine (GPC), myrystic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), arachidonic acid (20:4), monooleolylglycerol (MAG), diacylglycerol (DAG 18:1 and 16:0), triacylglycerol (TAG), tetramethylurea (TMU), PC 18:1, PC 16:0, PE 18:1, PE 18:0 and phosphatidic acid (PA) were kindly provided by Dr. M. Houweling from Dr. D. E. Vance's laboratory, Lipid and Lipoprotein Research Group, University of Alberta.

Bacterial Strains and Growth Conditions. *C. jejuni* strain UA580 and *H. pylori* UA763 were kindly provided by Dr. D. E. Taylor, Medical Microbiology and Immunology, University of Alberta. The organisms were stored in brain heart infusion

broth (BHI, Difco Laboratories) with 10% glycerol (BDH) at -70°C. Cultures were plated on supplemented blood agar plates (Triage Microbiological Systems) for 24 hours. The organisms were then inoculated into BHI broth and incubated overnight at 37°C. They were grown under microaerophilic conditions (i.e., 10% CO_2, 5% O_2, 85% N_2) with constant shaking. After centrifugation at 6000 rpm for 6 minutes, the bacteria were resuspended in BHI broth and used in further experiments.

Culture of Eukaryotic Cells. The Chinese hamster ovary cell line CHO-K1 (American Type Culture Collection (ATCC), CCL 61) and CHO cell sialic acid-deficient mutants Lec1 (ATCC CRL 1735), Lec2 (ATCC CRL 1736), and Lec8 (ATCC CRL 1737) were used. All cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. CHO cells were grown in Ham's F-12 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS). CHO cell mutants were grown in α-minimal essential media (MEM, Gibco) supplemented with 10% (v/v) FBS. Both CHO cells and CHO cell mutants were harvested by trypsinization and plated in a 24-well Multiwell tissue culture plate (Falcon). The phenotype of the CHO cell mutants was confirmed in a previous report (19).

Invasion and Binding Assays. Confluent cell monolayers were infected with approximately 10^9 organisms for 5 hours at 37°C in a humidified atmosphere. After infection, cells were washed with phosphate buffered saline (PBS) and incubated at 37°C for 1 hour with 100 mg/ml gentamicin (Gibco BRL) in FBS supplemented medium to kill any extracellular organisms. The cells were washed again and treated with 1% Triton X-100 (Sigma) in PBS at 37°C for 5 minutes releasing any intracellular organisms. Dilutions from each well were plated on BHI agar.

To determine the total number of organisms bound, the invasion assay was done at the same time as a modified version of the assay which excluded the gentamicin step. Since the modified version of the assay yielded number of organisms bound plus

internalized, the difference between total bound and internalized was taken to give the amount that were bound.

Wells containing uninfected cells were counted after each experiment to ensure that similar numbers of each cell type were infected.

Competitive Binding Assay with Lipid Vesicles. Since the lipids used in this study were not soluble in aqueous solutions, phosphatidylcholine / oleic acid vesicles were used in the competition assay. PC / oleate (1:1, molar ratio) vesicles were prepared as described by Weinhold *et al.* (52). Briefly, the mixture was evaporated under nitrogen and then sonicated into tissue culture medium.

Confluent CHO cell monolayers, in tissue culture medium with or without lipid vesicles, were infected with approximately 10^9 organisms for 5 hours on ice. The experiment was done on ice to avoid lipid incorporation and to prevent bacterial internalization. The cells were then washed with PBS and treated with 1% Triton X-100 (Sigma) in PBS for 5 minutes. Dilutions from each well were then plated on BHI agar.

Lipid Extraction from Eukaryotic Cells. For large scale extraction, CHO cells were grown to confluency in a T850 roller flask (Falcon), trypsinized and washed in PBS. The PBS was removed by centrifugation and the lipids were extracted by a modified method of Bligh and Dyer (2) by shearing in 1 ml of chloroform and 2 ml of methanol per 1 gram of wet cell weight. The mixture was filtered through a sintered glass funnel and the cells were sheared again in chloroform and refiltered. Then, 0.88% (w/v) potassium chloride (Fisher Scientific) in water was added to the two filtrates to remove non-lipid contaminants. The mixture was shaken and allowed to settle. The upper layer was removed and the lower layer was dried under nitrogen (6, 7).

Detection of Lipid Binding by TLC. The binding of *C. jejuni* UA580 to lipids was observed by TLC overlay according to the procedure of Lingwood *et al.* (27, 28). Briefly, lipid extracts were separated on silica coated plastic backed sheets (Polygram SIL-G, Macherey-Nagel) in chloroform/methanol/water (65:25:4, vol/vol/vol). The TLC overlay

was blocked with 3% gelatin (w/v, Difco Laboratories) in PBS at 37°C for 2 hours. The plate was then washed and incubated with *C. jejuni* UA580 at 37°C under microaerophilic conditions for 2 hours. The overlay was washed and incubated with antiserum overnight at 4°C. *C. jejuni* antiserum, used in this study, was raised in rabbits against the major outer membrane protein (MOMP, (47)). After washing with PBS, the overlay was incubated with alkaline phosphatase conjugated antibody for 1 hour at room temperature, washed with PBS, and developed with a NBT/BCIP detection system.

Staining of Lipids Separated by TLC. Lipid staining with iodine was performed to evaluate successful extraction and separation of lipids. Iodine vapors detect unsaturated compounds as well as several other compounds as yellow-brown zones. A parallel TLC plate was sprayed with 0.5% (w/v) α-naphthol in methanol/water (1:1, v/v), dried at room temperature, then sprayed with sulfuric acid/water (95:5, v/v), and heated to 120°C. This stain detects glycolipids as blue-purple zones, phospholipids as yellow zones, and cholesterol as grey-red zones (49).

Detection of Lipid Binding by ELISA. Lipid binding was quantitated by ELISA. Essentially, pure lipid species were serially diluted in chloroform or chloroform/methanol solutions. Once the solvents evaporated, the plates were washed and blocked with 3% gelatin (w/v) in PBS for 2 hours at 37°C. The plates were washed again and incubated with freshly cultured *C. jejuni* or *H. pylori* under microaerophilic conditions for 2 hours at 37°C. After washing, bound organisms were detected by using *C. jejuni* MOMP antiserum or *H. pylori* antiserum, obtained from a *H. pylori* positive volunteer, and an alkaline phosphatase detection system. Incubations in the absence of lipid were carried out simultaneously to account for any background binding and these values were subtracted from the reported absorbance readings.

3.1.3. RESULTS

We used CHO cell mutants with defined defects in complex carbohydrate biosynthesis (Figure 3.1) to investigate the role of oligosaccharide sequences in *C. jejuni* attachment to eukaryotic cells. If sialic acid was involved in the initial interaction between *C. jejuni* and the host cell, binding to the lectin mutants would be lower than to the parental sialylated CHO cells. However, binding to the lectin mutants did not decrease (Figure 3.2), suggesting that sialyllactosamine sequences are not important in *C. jejuni* binding. Interestingly, *C. jejuni* exhibited an increase in binding and invasion in Lec1 cells (Figure 3.2). Since this carbohydrate mutant terminates in mannose we speculated that *C. jejuni* may be recognizing mannose sequences on the host cell surface. We therefore tested whether mannose can inhibit binding of *C. jejuni* to parental CHO cells. Mannose, as well as several other simple sugars, did not significantly inhibit binding of *C. jejuni* to parental CHO cells (Figure 3.3).

Walan and Kihlström demonstrated that all strains of *C. jejuni* were negatively charged and exposed a hydrophobic surface (50). In light of the carbohydrate findings, we speculated that *C. jejuni* may be interacting hydrophobically with cellular lipids. Perhaps increases in *C. jejuni* binding to Lec1 cells may not be dependent on the sugar structure; the Lec1 mutation may allow *C. jejuni* to make better contact with the cell surface due to the presence of truncated carbohydrate sequences. We therefore extracted the lipids from CHO cells to determine whether *C. jejuni* could bind to lipids found in the cell membrane.

The main lipids extracted from CHO cells, listed in the order of increasing retention time, were: glycolipids, including gangliosides; LysoPE; PC; PE; the breakdown product of cardiolipin; cardiolipin; cholesterol and more mobile neutral lipids at the solvent front (fatty acids and acylglycerols). In the α-naphthol stained TLC, the component in the extract that demonstrated the same R_f as cholesterol stained red confirming that it is cholesterol. Spots that did not show any streaking (ran parallel with the solvent front) stained purple, indicating that they are glycolipids. All remaining spots were yellow-brown indicating that they are phospholipids. TLC overlay results showed that *C. jejuni* bound

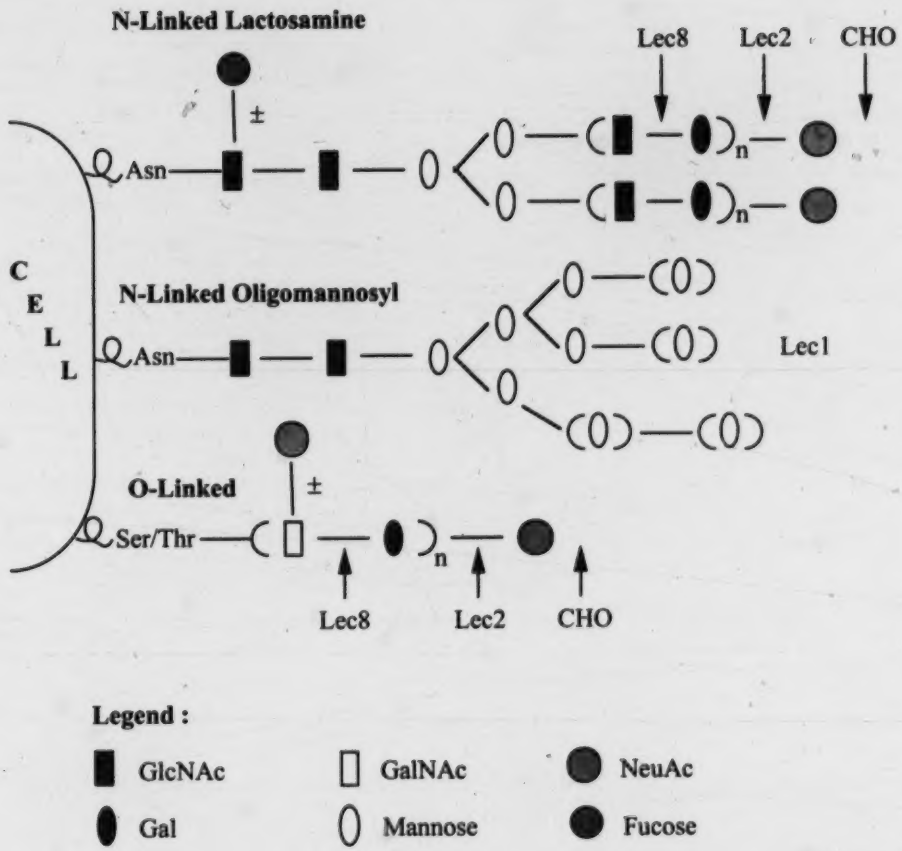


Figure 3.1. Schematic diagram of carbohydrate structures associated with parental CHO cells and CHO cell mutants adapted from P. Stanley [Stanley, 1989 #62]. Although only glycoprotein mutations are illustrated, similar carbohydrate mutations occur in the glycolipids of Lec2 and Lec8 cells.

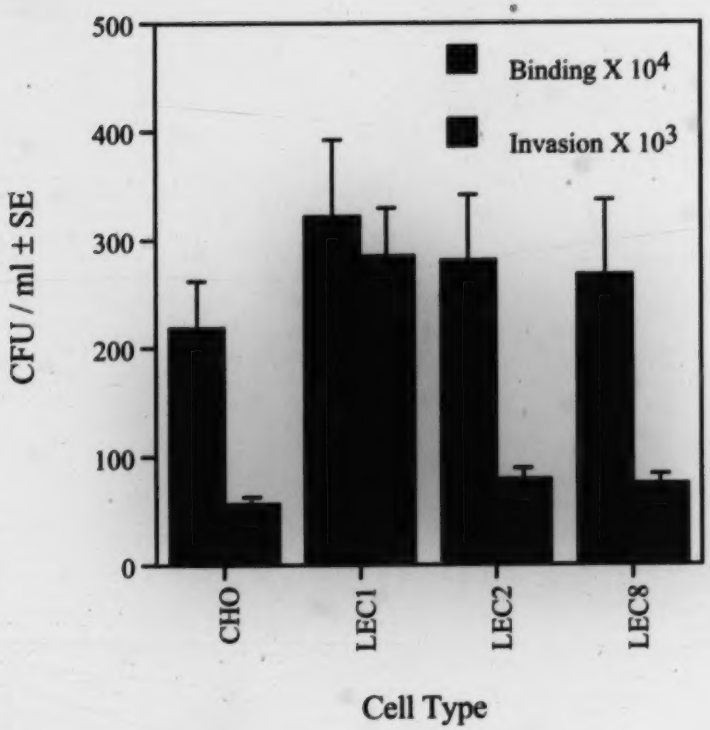


Figure 3.2. Comparison of *C. jejuni* binding and invasion of CHO cells and CHO cell mutants. Experiments were done as described in duplicate (binding) or triplicate (invasion). The colony forming units were determined per ml ± standard error (SE). P values of <0.05 were considered significant by the t-test.

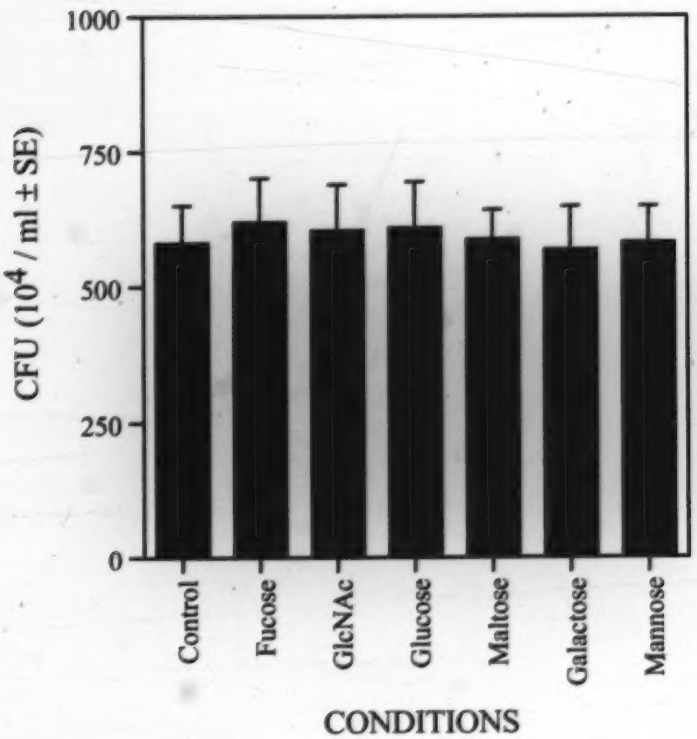


Figure 3.3. *C. jejuni* binding to CHO cells in the presence of simple carbohydrates. Concentrations of 0.5% (w/v) fucose, N-acetylglucosamine (GlcNAc), glucose, maltose, galactose or mannose in Ham's F-12 medium/FBS were added to each well. Control wells contained only tissue culture medium. Experiments were done as described in duplicate. The colony forming units were determined per ml ± SE. P values of >0.05 were considered to be not significant by the t-test.

strongly to LysoPE, PC, PE, cardiolipin and to its breakdown product and with less affinity to glycolipids, cholesterol and the more mobile neutral lipids (Figure 3.4).

We then quantitated and extended these findings by ELISA using lipid standards. Table 3.1 shows that *C. jejuni* binds to all lipids tested. The concentration of lipid required for maximum binding was between 60-100 ng/well (85-142 pmol/well). Alternatively, cholesterol concentrations, greater than 30 μg/well, were required for maximal binding of *C. jejuni*. For comparison, the lipid binding ELISA was also done with *H. pylori* UA763. Table 3.1 shows that *H. pylori* also binds to all the lipids including cholesterol but, at concentrations greater than 30 μg/well.

Since *C. jejuni* bound to all the lipids equally, we speculated that the binding was mediated by nonspecific hydrophobic interactions between *Campylobacter* and the fatty acid tails. Various concentrations of tetramethylurea (TMU), a strong disrupting agent of hydrophobic interactions, were co-incubated with *C. jejuni* in the presence of 100 ng PC in the ELISA assay. As shown in Figure 3.5, TMU concentrations as high as 1.0 M did not significantly alter the binding of *C. jejuni* to PC. Bacterial viability was not affected by TMU (not shown).

We further dissected the interaction by comparing *Campylobacter* binding to lipids and to lipid derivatives. Representative ELISA results, shown in Figure 3.6, demonstrate that the removal of any portion of the lipid molecule resulted in a decrease in the binding affinity. *C. jejuni* binding to PC was unaltered regardless of the saturation or length of the fatty acid tails. However, a ten-fold higher concentration of monoacyl-PC (LysoPC) was required to show the same binding kinetics as PC while a 100-fold higher concentration of PA and greater than a 100-fold higher concentration of deacylated PC (GPC) was required to show the same results. These observations are summarized in the schematic shown in Figure 3.6.

The same analysis was also done by TLC overlay (Figure 3.7). Interestingly, binding to lipids was dependent on the fatty acid composition. *C. jejuni* binding by TLC

Figure 3.4. Analysis of lipids extracted from CHO cell membranes. The extracted sample (S) was spotted at the indicated concentration (μg total lipid). A) TLC plate, stained with α-naphthol, comparing the retention times of lipid standards to the lipid extact. B) Same as in A) but stained with iodine. C) TLC overlay demonstrating concentration dependent *C. jejuni* binding to lipids extracted from CHO cells.

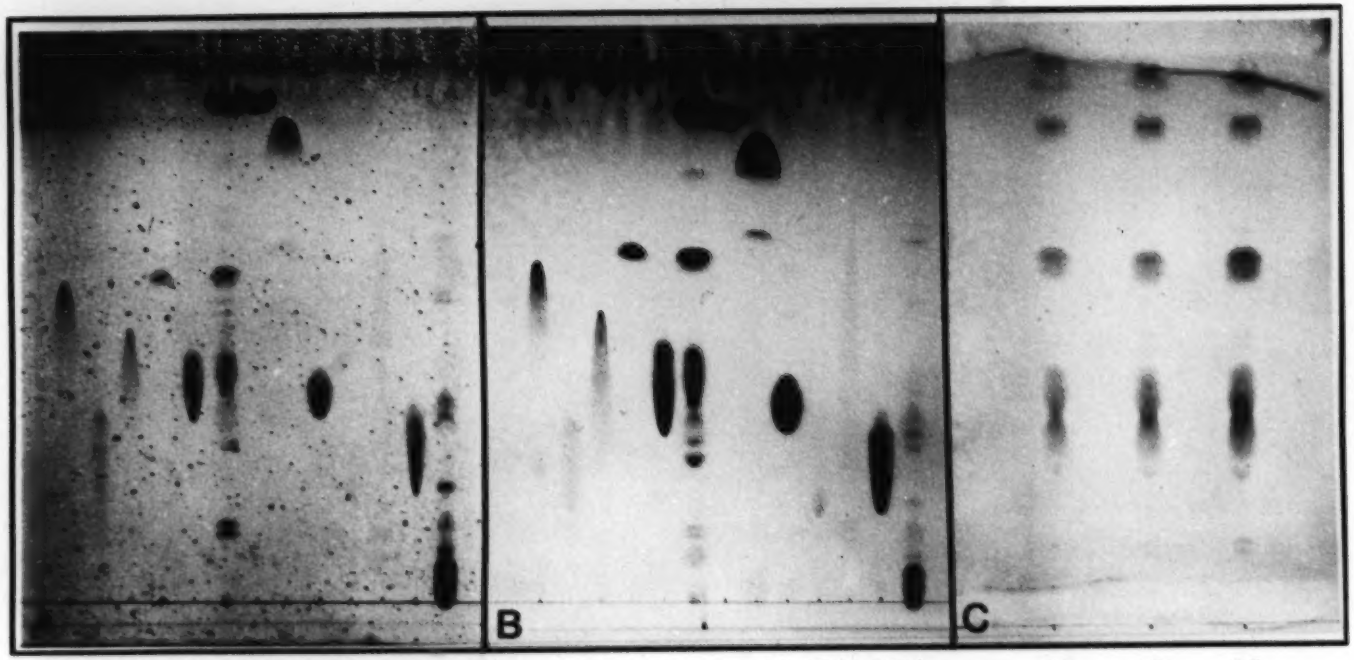

B
C
PG PI PS PE PC S80 Ch Ca LPE LPC PA SP Ga
PG PI PS PE PC S80 Ch Ca LPE LPC PA SP Ga
S33
S50
S67

Table 3.1. Comparison of lipid concentrations required to saturate binding of *C. jejuni* UA580 and *H. pylori* UA763.

LIPID	*C. jejuni* UA580	*H. pylori* UA763
Cholesterol	31 300 ± 873 ng/well	200 000 ± 10 000 ng/well
Phosphatidylcholine	62.5 ± 1.34 ng/well	40 000 ± 100 ng/well
Phosphatidylethanolamine	62.5 ± 0.00 ng/well	200 000 ± 20 000 ng/well
Phosphatidylglycerol	125 ± 8.62 ng/well	40 000 ± 70 ng/well
Phosphatidylserine	62.5 ± 5.38 ng/well	200 000 ± 100 ng/well
Phosphatidylinositol	125 ± 3.96 ng/well	40 000 ± 2 000 ng/well
Sphingomyelin	125 ± 3.81 ng/well	200 000 ± 3 000 ng/well

Note: *H. pylori* concentrations were determined from 1/5 fold lipid dilutions. Therefore, 40 000 ng/well and 200 000 ng/well absorbance readings differed by one well. *C. jejuni* concentrations were determined from 1/2 fold lipid dilutions. Therefore, 62.5 ng/well and 125 ng/well absorbance readings differed by one well.

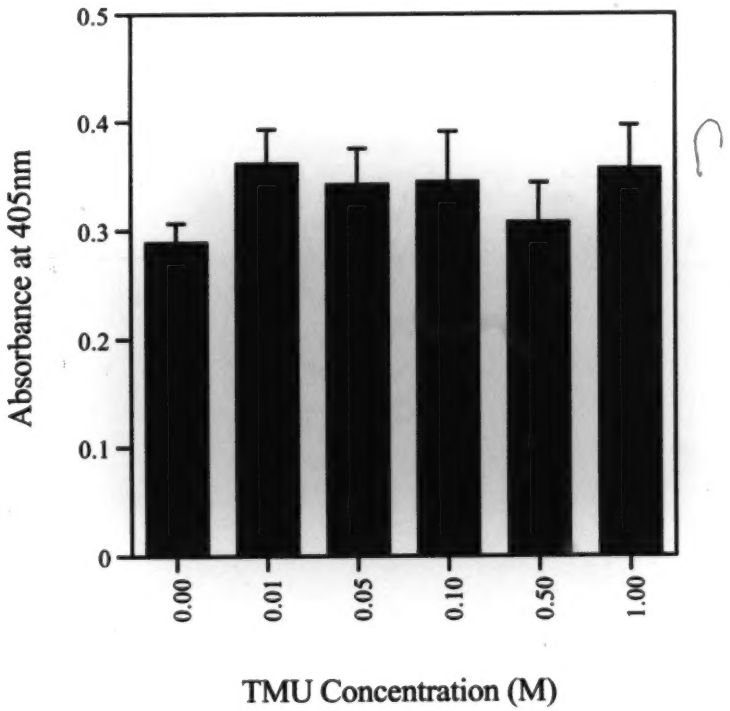


Figure 3.5. Affect of varying concentrations of tetramethylurea on the binding of *C. jejuni* to ELISA wells coated with 100 ng PC. P values of <0.05 were considered significant.

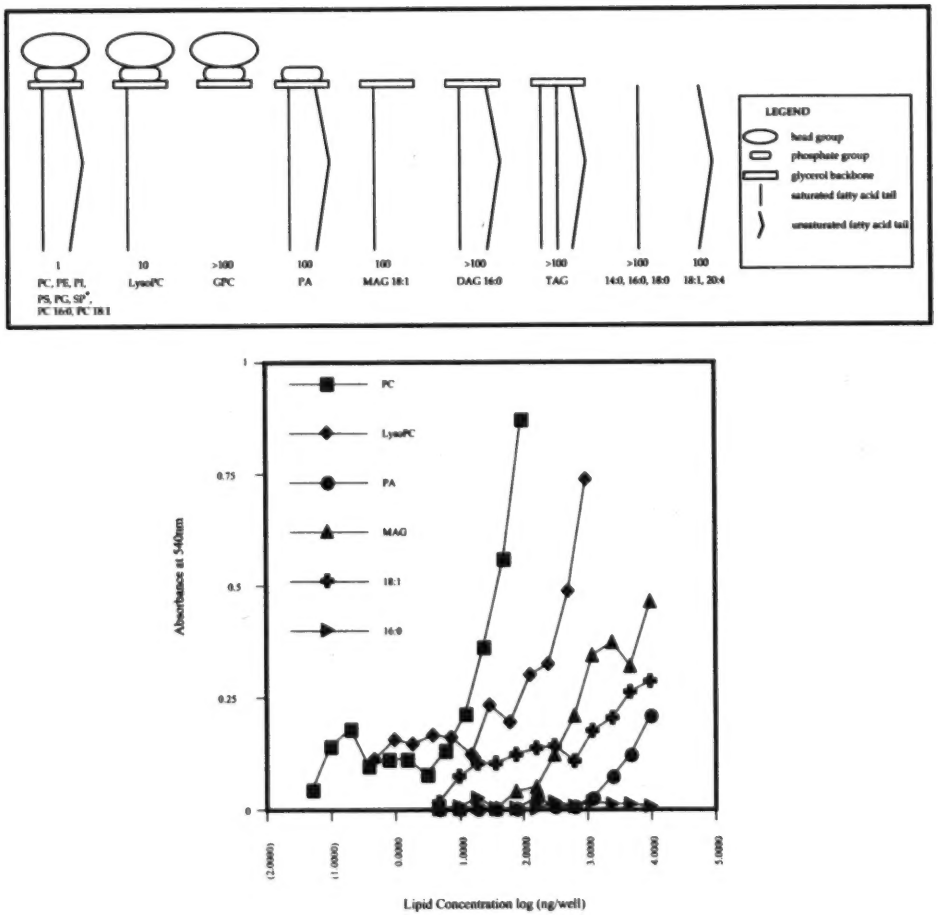


Figure 3.6. Schematic diagram illustrating the relative affinities of *C. jejuni* for lipids and lipid derivatives. Lipids were assigned a value of 1. Relative concentrations of lipid derivatives needed for *C. jejuni* binding are shown. A representative graph demonstrating results obtained by Elisa is also shown.

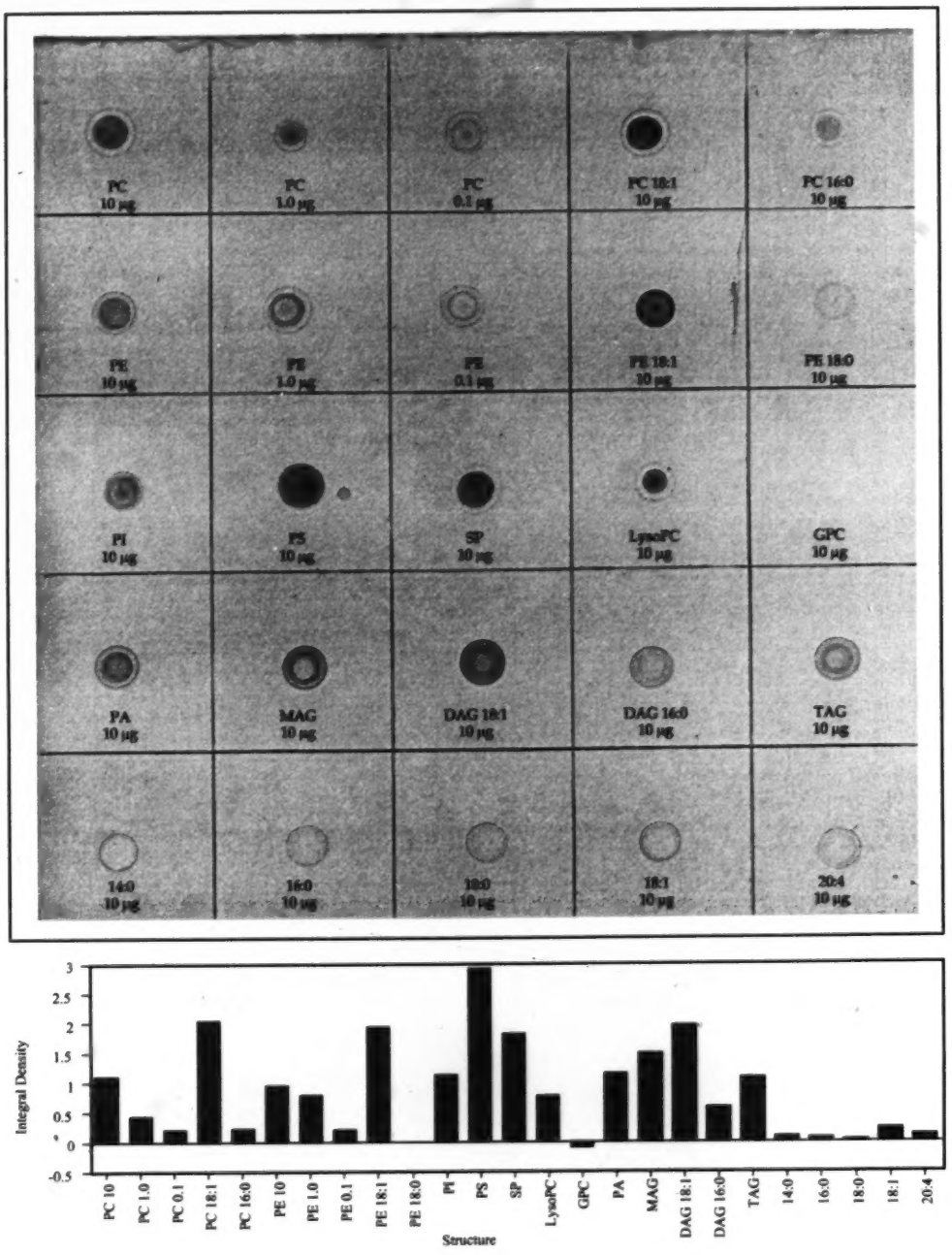


Figure 3.7. Modified TLC overlay. Known amounts of lipids and lipid derivatives were spotted. The overlay demonstrates *C. jejuni* affinity for unsaturated fatty acid chains which was not evident with the ELISA procedure. The color intensity of each spot, in a constant area, was measured and plotted as the integral density.

overlay showed the following trend: PC 18:1 > heterogeneous PC > PC 16:0 and PE 18:1 > heterogeneous PE > PE 18:0 (Figure 3.7). Contrary to what we expected, the results suggest that *C. jejuni* binds specifically to the entire lipid molecule.

C. jejuni binding to CHO cell monolayers was significantly inhibited by 1 mM PC / oleate vesicles (Figure 3.8). However, *C. jejuni* binding could not be further inhibited by increasing concentrations of lipid vesicles.

3.1.4. DISCUSSION

One of the least understood aspects of *Campylobacter* virulence is the interaction between these organisms and intestinal cells. Adhesion may be considered the first step in the interaction between *C. jejuni* and the intestinal cell. Without attachment *C. jejuni* cannot colonize and will be quickly removed by nonspecific host defense mechanisms. Several investigators have tried to demonstrate a role for carbohydrates as receptors for *C. jejuni*. The reports have been inconclusive (32-34). We investigated the role of carbohydrate sequences by comparing binding and invasion of *C. jejuni* to CHO cells and their lectin mutants. The high mannose $(Man)_5(GlcNAc)_2$-Asn M5 structure (Figure 3.1) is the starting point for the biosynthesis of all complex N-linked sugars (41). The Lec1 mutation results in a high mannose phenotype on all N-linked sugars. In Lec2 and Lec8 mutants both glycoproteins (N- and O- linked) and glycolipids are synthesized with truncated carbohydrates (42). In our experiments with CHO cell mutants, we could not demonstrate a role for the carbohydrate sequences examined. We also could not alter the binding of *C. jejuni* to CHO cells with fucose, N-acetylglucosamine, glucose, maltose, galactose, or mannose. However, we have not ruled out the possibility that yet unidentified complex carbohydrates may play a role in attachment of *C. jejuni* to host cells. Such unidentified interactions may be responsible for the residual *C. jejuni* binding observed in lipid competition experiments (Figure 3.8).

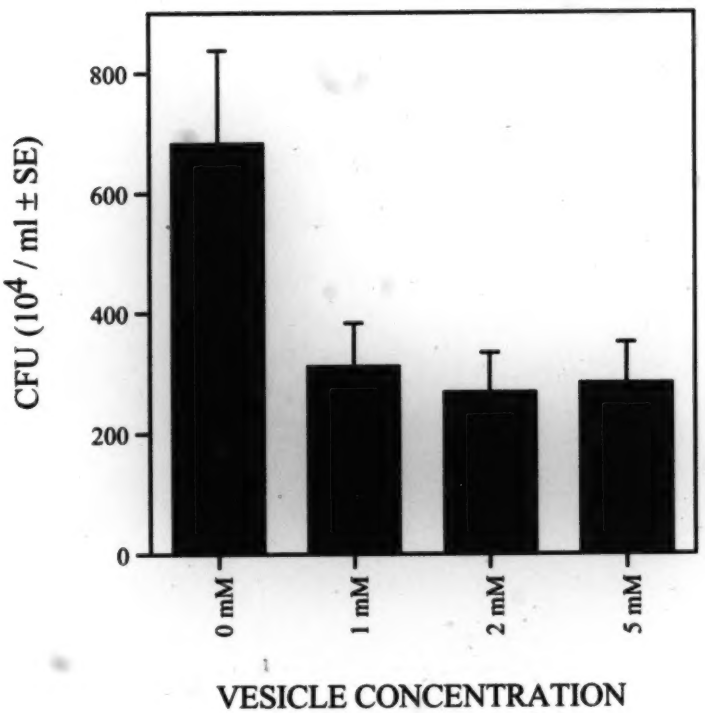


Figure 3.8. *C. jejuni* binding to CHO cells in the presence of PC/oleate vesicles. Control wells contained only tissue culture medium. Experiments were done as described in triplicate. The colony forming units were determined per ml ± SE. P values of <0.05 were considered significant.

Walan and Kihlström demonstrated that all strains of *C. jejuni* were negatively charged and exposed a hydrophobic surface (50). Kervella *et al.* showed that adherence of *C. jejuni* was markedly reduced by detergents suggesting hydrophobic interactions participate in adherence (20). Lingwood *et al.* demonstrated that a closely related organism, *H. pylori,* binds specifically to PE. These observations prompted us to investigate whether *Campylobacter* interacted hydrophobically with membrane lipids. As can be seen in Figure 3.4, *C. jejuni* bound to several lipids extracted from CHO cells suggesting a biological role for these lipids in adhesion.

C. jejuni bound to LPE, PC, PE and cardiolipin extracted from CHO cells. *Campylobacter* also bound to extracted glycolipids and neutral lipids but the color intensity on TLC overlay was greatly reduced relative to that seen for phospholipids. The observation that carbohydrates on lipids as well as on proteins play a subordinate role in binding is further supported by our observation that *Campylobacter* binding to CHO cell mutants with truncated lipid carbohydrates (Lec2 and Lec8) was not significantly different from binding to parental CHO cells with intact glycolipids.

In ELISA experiments, *C. jejuni* bound with high affinity to PE, PC, PI, PS, PG, and SP but, not to cholesterol. Amphiphilic lipids can be broken down into two groups: glycerolipids which include phospholipids and sphingolipids which include sphingomyelin. Since *C. jejuni* bound to phospholipids and SP, we describe the event as lipid binding. Although, SP exhibits noticeable differences in structure, space-filling and electron cloud models of SP and PC show that the two lipids have very similar three dimensional conformations (45). The observation that *C. jejuni* bound to SP and PC with similar affinities suggests that the three dimensional conformation may be important for binding. It is also of significance that *C. jejuni* can bind to both choline lipids which predominate in the outer leaflet of the cell membrane (18) suggesting a biological role for these lipids.

Investigating this interaction further confirmed that *C. jejuni* did not solely interact hydrophobically with the fatty acid tails of the lipids assayed. Concentrations of TMU as high as 1.0 M did not significantly inhibit binding of *C. jejuni* to wells coated with PC. In ELISA assays concentrations as low as 0.05 M TMU inhibited binding of *Pseudomonas aeruginosa* to mucin and to BSA (40). Binding of *Yersinia enterocolitica* to delipidated mucins was prevented by 0.5 M concentrations of TMU (29). Treatment with 0.5 M TMU was also shown to reduce binding of *Y. enterocolitica* to polystyrene by 80% (30). Finally, TMU concentrations as low as 0.05 M reduced adherence of *Streptococcus salivarius* to saliva coated hydroxyapatite (51).

Both TLC overlay and ELISA demonstrated that removal of any portion of the lipid molecule decreased *C. jejuni* binding affinity. *C. jejuni* bound specifically to the whole lipid molecule showing preference, in the TLC overlay procedure, for unsaturated fatty acid tails. Other groups have also noted discrepancies in binding affinities due to the physical mode of lipid immobilization (3, 11, 37, 43).

Since the TLC plates, used in this study, consist of silica coated onto plastic sheets, the primary interaction with any adsorbed components would be hydrophilic. Phospholipids would interact mainly with their polar head groups when coated onto the silica gel. Therefore, the fatty acid tails may be more accessible allowing *Campylobacter* adhesins to differentiate between saturated and unsaturated hydrocarbons. In contrast, polypropylene ELISA plates would primarily interact hydrophobically with the fatty acid tails of the coated lipids. Indeed, phospholipids have been described to form a monolayer when coated onto hydrophobic polystyrene plates (11). Alternatively, lipids have been shown to form bilayers on glass microspheres (11). Since glass and silica gel exhibit similar chemical properties, it is possible that lipids may also form bilayers on silica coated TLC plates. In this model, the fatty acid tails would also be directed away from *Campylobacter* suggesting that the presentation and packing of the lipid may be more important for binding than interaction with the fatty acid tails.

Differences in binding due to fatty acid saturation has also been described by several groups. Binding of *E. coli* verotoxin to glycosphingolipids was altered depending on the fatty acid moiety (3, 21). The observation that *H. pylori* bound variably to PE from different sources also suggested that the nature of the fatty acids was important (13). Interestingly, *H. pylori* bound PE from *E. coli* with the highest affinity (13, 27). Fatty acids found in prokaryotes consist of saturated and monounsaturated hydrocarbon chains as well as branched chains and chains containing cyclopropane (15). Since differences in hydrophobicity would be minimal between a saturated hydrocarbon, a chain with one unsaturation, a chain with one branching and a chain with a cyclopropane group, we believe that the spacial conformation of the lipid is important for binding to its receptor. We also observed greater binding of *Campylobacter* to PE isolated from *E. coli* even when compared to PE 18:1 (unpublished observation).

Although we were unable to confirm the PE binding specificity described by Lingwood *et al.*, *C. jejuni* appears to display an overall higher affinity for lipids than *H. pylori*. We speculate that lipid-binding proteins may be present on the outer membrane of *C. jejuni*. These proteins would be capable of binding to a wide variety of lipids. The binding affinity of these proteins would be dependent upon the three dimensional conformation of the lipid. However, the aminophospholipids, PE and PS, predominate in the inner leaflet of the cell membrane (18), thus *C. jejuni* may not be able to bind to all of the lipids described *in vivo*.

Since the genome of *C. jejuni* is only half the size of that of *E. coli* or *Salmonella* (5), *C. jejuni* may use one protein for multiple purposes. The lipid-binding protein may act as an adhesin as well as possibly playing a role in lipid transport or lipolysis. Indeed, Pei and Blaser identified another protein of *C. jejuni*, PEB1, and showed that PEB1 played a role in adherence and metabolism (36). We speculate that adherence to lipids is secondary and that the interaction described in this study has a yet unidentified role *in vivo*. We are currently isolating the bacterial adhesin involved in this interaction.

3.2. RELATED WORK

When we initially discovered that *C. jejuni* interacts with all phospholipids equally, we speculated that binding occurred through nonspecific hydrophobic interactions between *Campylobacter* and the fatty acid tails. Therefore, we measured *C. jejuni* cell surface hydrophobicity in the hexadecane test and compared *C. jejuni* surface hydrophobicity with that of *S. choleraesuis*. We also confirmed that *C. jejuni* binds to the phospholipids and that the reported binding results were not due to nonspecific binding of *C. jejuni* anti-MOMP antibody or anti-rabbit secondary antibody binding to phospholipids. Finally we describe BSA interference in *C. jejuni* binding to lipids. This observation suggested that we perform the lipid vesicle inhibition experiments without BSA-containing FBS.

Determination of hydrophobicity. *C. jejuni* UA580 and ER1109 and *S. choleraesuis* SL2824 overnight cultures were concentrated by centrifugation. Hydrophobicity was determined according to the method of Rosenberg *et al.* (39). The cell pellets were washed twice with 10 ml of PUM buffer (97 mM K_2HPO_4, 53 mM KH_2PO_4, 30 mM urea, 0.8 mM $MgSO_4 \cdot 7H_2O$, pH 7.1). The pellets were then resuspended in 3 ml of PUM buffer and diluted 1:6 (vol/vol). The optical density was measured at 550 nm. Then 300 μl of hexadecane was added and the mixture was stirred for 1 min. After two hours, the optical density was read again and the percent decrease in optical density was recorded. In this experiment, a decrease in optical density is a reflection of the affinity of the organism for the hydrophobic solvent hexadecane. From the results presented in Table 3.2 it can be concluded that *C. jejuni* is somewhat hydrophobic since a portion of *C. jejuni* partitioned into the hydrophobic phase. Also, the two *C. jejuni* strains examined are more hydrophobic than *S. choleraesuis* SL2824. Indeed, Walan and Kilhström also reported that all the strains of *C. jejuni* they examined exposed a hydrophobic surface (50). However, in comparison to several other microorganisms, *C. jejuni* hydrophobicity is rather low (39). For example, *Acinetobacter calcoaceticus* shows a greater than 75% decrease in optical density in the hexadecane test (39). It is of interest that relative

TABLE 3.2. Determination of *C. jejuni* UA580, *C. jejuni* ER1109, and *S. cholerasuis* SL2824 hydrophobicity.

Isolate	OD* at start	OD at 2 hrs	Decrease in OD (%)
C. jejuni UA580	0.573	0.520	9.25
C. jejuni ER1109	0.620	0.560	9.68
S. choleraesuis SL2824	0.245	0.245	0.00

*OD represents the optical density at 550nm

Note: The results shown in the table are from one experiment.

hydrophobicities and surface charge have been shown to play roles in bacterial adherence and susceptibility to phagocytosis as well as in agglutination and pathogenesis (12, 16, 48, 50). The hexadecane test is a rapid and commonly used assay (16, 39). However, the test is not always reliable (16).

Control experiments. To determine background levels of binding in the lipid microtiter assay, phosphatidylcholine (PC) coated wells were incubated with or without *C. jejuni* UA580 followed by incubations with various dilutions of the primary antibody, *C. jejuni* UA580 anti-MOMP. It was determined that binding in the ELISA assay was dependent on the presence of bacteria and not just a result of interactions between the lipid and the primary or secondary antibodies. Also, a primary antibody dilution of 1:500 (in comparison to 1:1000 and 1:18000) was determined to be sensitive enough to detect bacterial binding to wells containing as little as 7.9 ng of PC compared to wells without bacteria (1:500 w/o Cj) and wells without PC (blank), (Figure 3.9). These observations formed the basis for the numerous ELISA experiments described in this chapter.

Albumin is an abundant protein responsible for approximately 50% of total plasma proteins in humans. Since each molecule of bovine serum albumin (BSA) can bind up to 10 molecules of free fatty acid, we wanted to determine if BSA could inhibit *C. jejuni* UA580 binding to PC-coated microtiter wells. BSA, at a concentration of 50 μM, inhibited *C. jejuni* binding to PC by approximately 50% ($p=0.002$). As a result of this observation, serum albumin was removed from the tissue culture media in experiments demonstrating lipid vesicle inhibition of *C. jejuni* binding to Caco-2 cells.

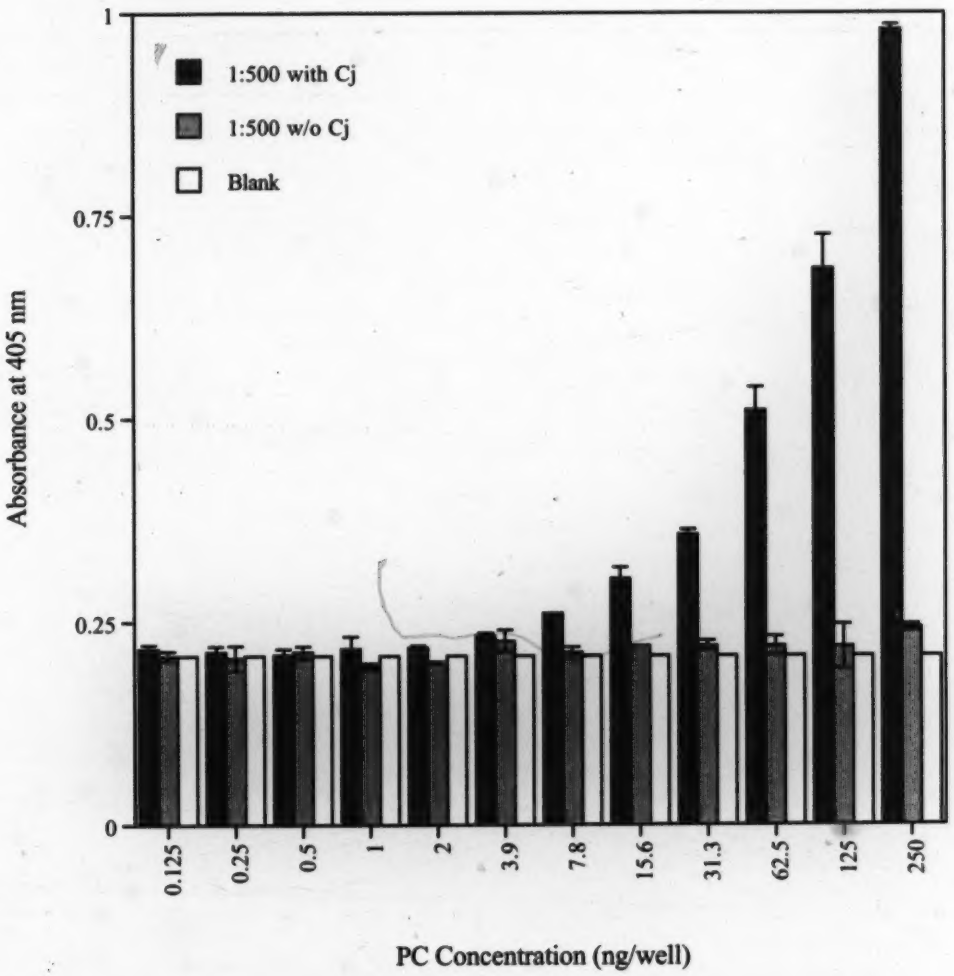


Figure 3.9. Binding of *C. jejuni* UA580 Specifically to PC. The data shown were determined from one ELISA experiment. Cj represents *C. jejuni* UA580. w/o represents without *C. jejuni*.

3.3. REFERENCES

1. **Backenson, P. B., J. L. Coleman, and J. L. Benach**. 1995. *Borrelia burgdorferi* shows specificity of binding to glycosphingolipids. Infect. Immun. **63:** 2811-2817.

2. **Bligh, E. G., and W. J. Dyer**. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:** 911-917.

3. **Boyd, B., G. Magnusson, Z. Zhiuyan, and C. A. Lingwood**. 1994. Lipid modulation of glycolipid receptor function-availability of Gal(α1-4)Gal disaccharide for verotoxin binding in natural and synthetic glycolipids. Eur. J. Biochem. **223:** 873-878.

4. **Brennan, M. J., J. H. Hannah, and E. Leininger**. 1991. Adhesion of *Bordetella pertussis* to sulfatides and to the GalNAc beta 4Gal sequence found in glycosphingolipids. J. Biol. Chem. **266:** 18827-18831.

5. **Chang, N., and D. E. Taylor**. 1990. Use of pulsed-field agarose gel electrophoresis to size genomes of *Campylobacter* species and to construct a *Sal*I map of *Campylobacter jejuni* UA580. J. Bacteriol. **172:** 5211-5217.

6. **Christie, W. W.** 1982. The isolation of lipids from tissues. p. 17-23. *In* Lipid Analysis. Pergamon Press, Oxford .

7. **Christie, W. W.** 1984. Extraction and hydrolysis of lipids and some reactions of their fatty acid components, p. 33-46. *In* Mangold, H. K. (ed.), Lipids. CRC Press, Boca Raton .

8. **Cinco, M., E. Banfi, E. Ruaro, D. Crevatin, and D. Crotti**. 1984. Evidence for L-fucose (6-deoxy-L-galactopyranose)-mediated adherence of *Campylobacter* spp. to epithelial cells. FEMS Microbiol. Letters. **21:** 347-351.

9. **Deal, C. D., and H. C. Krivan**. 1990. Lacto- and ganglio-series glycolipids are adhesion receptors for *Neisseria gonorrhoeae*. J. Biol. Chem. **265:** 12774-12777.

10. **deMelo, M. A., and J. Pechere**. 1990. Identification of *Campylobacter jejuni* surface proteins that bind to eucaryotic cells *in vitro*. Infect. Immun. **58:** 1749-1756.

11. **Eschwège, V., I. Laude, F. Toti, J. L. Pasquali, and J. M. Freyssinet**. 1996. Detection of bilayer phospholipid-binding antibodies using flow cytometry. Clin. Exp. Immunol. **103:** 171-175.

12. **Field, L. H., J. L. Underwood, S. M. Payne, and L. J. Berry**. 1993. Characteristics of an avirulent *Campylobacter jejuni* strain and its virulence-enhanced variants. J. Med. Microbiol. **38:** 293-300.

13. **Gold, B. D., M. Dytoc, M. Huesca, D. Philpott, A. Kuksis, S. Czinn, C. A. Lingwood, and P. M. Sherman**. 1995. Comparison of *Helicobacter mustelae* and *Helicobacter pylori* adhesion to eukaryotic cells *in vitro*. Gastroenterol. **109:** 692-700.

14. **Gold, B. D., M. Huesca, P. M. Sherman, and C. A. Lingwood**. 1993. *Helicobacter mustelae* and *Helicobacter pylori* bind to common lipid receptors *in vitro*. Infect. Immun. **61:** 2632-2638.

15. **Goldfine, H.** 1982. Lipids of prokaryotes-structure and distribution, p. 1-43. *In* Razin, S., and S. Rottem (eds.), Membrane lipids of prokaryotes. Academic Press Inc., New York .

16. **Goldstein, B., S. Magdassi, and J. S. Rokem**. 1993. Surface angle of bacterial colony is directly correlated to hydrophobicity of bacterium. Microbios. **75:** 181-184.

17. **Goodwin, C. S., J. A. Armstrong, T. Chilvers, M. Peters, M. D. Collins, L. Sly, W. McConnell, and W. E. S. Harper**. 1989. Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. Int. J. Sys. Bacteriol. **39:** 397-405.

18. **Hauser, H., and G. Lipka**. 1994. Lipid dynamics in brush border membrane, p. 167-195. *In* Hoekstra, D. (ed.), Cell lipids. Academic Press Inc., San Diego .

19. **Heerze, L. D., C. G. Clark, Y. Chen, R. H. Smith, and G. D. Armstrong**. 1991. Synthesis and characterization of a pertussis toxin-biotin conjugate. Biochem. Biophys. Res. Commun. **179:** 1464-1469.

20. **Kervella, M., J.-M. Pages, Z. Pei, G. Grollier, M. J. Blaser, and J.-L. Fauchere**. 1993. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. Infect. Immun. **61:** 3440-3448.

21. **Kiarash, A., B. Boyd, and C. A. Lingwood**. 1994. Glycosphingolipid receptor function is modified by fatty acid content-verotoxin 1 and verotoxin 2c

preferentially recognize different globotriaosyl ceramide fatty acid homologues. J. Biol. Chem. **269:** 11138-11146.

22. **Krivan, H. C., B. Nilsson, C. A. Lingwood, and H. Ryu**. 1991. *Chlamydia trachomatis* and *Chlamydia pneumoniae* bind specifically to phosphatidylethanolamine in Hela cells and to GalNAcβ1-4Glc sequences found in asialo-GM_1 and asialo-GM_2. Biochem. Biophys. Res. Commun. **175:** 1082-1089.

23. **Krivan, H. C., L. D. Olson, M. F. Barile, V. Ginsburg, and D. D. Roberts**. 1989. Adhesion of *Mycoplasma pneumoniae* to sulfated glycolipids and inhibition by dextran sulfate. J. Biol. Chem. **264:** 9283-9288.

24. **Kuusela, P., A. P. Moran, T. Vartio, and T. U. Kosunen**. 1989. Interaction of *Campylobacter jejuni* with extracellular matrix components. Biochim. Biophys. Acta. **993:** 297-300.

25. **Leffler, H., and C. Svanborg-Edén**. 1981. Glycolipid receptors for uropathogenic *Escherichia coli* on human erythrocytes and uroepithelial cells. Infect. Immun. **34:** 920-929.

26. **Lingwood, C. A., M. Cheng, H. C. Krivan, and D. Woods**. 1991. Glycolipid receptor binding specificity of exoenzyme S from *Pseudomonas aeruginosa*. Biochem. Biophys. Res. Commun. **175:** 1076-1081.

27. **Lingwood, C. A., M. Huesca, and A. Kuksis**. 1992. The glycerolipid receptor for *Helicobacter pylori* (and exoenzyme S) is phosphatidylethanolamine. Infect. Immun. **60:** 2470-2474.

28. **Lingwood, C. A., H. Law, A. Pellizzari, P. Sherman, and B. Drumm**. 1989. Gastric glycerolipid as a receptor for *Campylobacter pylori*. Lancet. **2:** 238-241.

29. **Mantle, M., and S. D. Husar**. 1994. Binding of *Yersinia enterocolitica* to purified, native small intestinal mucins from rabbits and humans involves interactions with the mucin carbohydrate moiety. Infect. Immun. **62:** 1219-1227.

30. **Mantle, M., and S. D. Husar**. 1993. Adhesion of *Yersinia enterocolitica* to purified rabbit and human intestinal mucin. Infect. Immun. **61:** 2340-2346.

31. **McSweegan, E., and R. I. Walker**. 1986. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect. Immun. **53:** 141-148.

32. **Moser, I., and E. Hellmann**. 1989. *In vitro* binding of *Campylobacter jejuni* surface proteins to murine small intestinal cell membranes. Med. Microbiol. Immunol. **178:** 217-228.

33. **Moser, I., W. F. K. J. Schröder, and E. Hellmann**. 1992. *In vitro* binding of *Campylobacter jejuni/coli* outer membrane preparations to INT 407 cell membranes. Med. Microbiol. Immunol. **180:** 289-303.

34. **Næss, V., A. C. Johannessen, and T. Hofstad**. 1988. Adherence of *Campylobacter jejuni* and *Campylobacter coli* to porcine intestinal brush border membranes. APMIS. **96:** 681-687.

35. **Olsen, G. J., C. R. Woese, and R. Overbeek**. 1994. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. **176:** 1-6.

36. **Pei, Z., and M. J. Blaser**. 1993. PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems. J. Biol. Chem. **268:** 18717-18725.

37. **Pellizzari, A., H. Pang, and C. A. Lingwood**. 1992. Binding of verotoxin 1 to its receptor is influenced by differences in receptor fatty acid content. Biochem. **31:** 1363-1370.

38. **Roberts, D. D., L. D. Olson, M. F. Barile, V. Ginsburg, and H. C. Krivan**. 1989. Sialic acid-dependent adhesion of *Mycoplasma pneumoniae* to purified glycoproteins. J. Biol. Chem. **264:** 9289-9293.

39. **Rosenberg, M., D. Gutnick, and E. Rosenberg**. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9:** 29-33.

40. **Sajjan, U., J. Reisman, P. Doig, R. T. Irvin, G. Forstner, and J. Forstner**. 1992. Binding of nonmucoid *Pseudomonas aeruginosa* to normal human intestinal mucin and respiratory mucin from patients with cystic fibrosis. J. Clin. Invest. **89:** 657-665.

41. **Schachter, H.** 1984. Coordination between enzyme specificity and intracellular compartmentation in the control of protein-bound oligosaccharide biosynthesis. Biol. Cell. **51:** 133-146.

42. **Stanley, P.** 1989. Chinese hamster ovary cell mutants with multiple glycosylation defects for production of glycoproteins with minimal carbohydrate heterogeneity. Mol. Cell. Biol. **9:** 377-383.

43. **Stewart, M. W., W. S. Etches, A. S. Russell, J. S. Percy, C. A. Johnston, C. K. Chew, and P. A. Gordon**. 1993. Detection of antiphospholipid antibodies by flow cytometry: rapid detection of antibody isotype and phospholipid specificity. Thromb. Haemostas. **70:** 603-607.

44. **Stromberg, N., C. Deal, G. Nyberg, S. Normark, M. So, and K.-A. Karlsson**. 1988. Identification of carbohydrate structures that are possible receptors for *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. USA. **85:** 4902-4906.

45. **Sweeley, C. C.** 1994. Lipids and the modulation of cell function, p. 357-360. *In* Hoekstra, D. (ed.), Cell lipids. Academic Press Inc., San Diego .

46. **Szymanski, C. M., M. King, M. Haardt, and G. D. Armstrong**. 1995. *Campylobacter jejuni* motility and invasion of Caco-2 cells. Infect. Immun. **63:** 4295-4300.

47. **Taylor, D. E., and N. Chang**. 1987. Immunoblot and enzyme-linked immunosorbent assays of *Campylobacter* major outer-membrane protein and application to the differentiation of *Campylobacter* species. Mol. Cell. Probes. **1:** 261-274.

48. **Van Oss, C. J., and C. F. Gillman**. 1972. Phagocytosis as a surface phenomenon: contact angles and phagocytosis of non-opsonized bacteria. J. Reticuloendothel. Soc. **12:** 283-292.

49. **Vioque, E.** 1984. Spray reagents for thin-layer chromatography (TLC) and paper chromatography (PC), p. 309-317. *In* Mangold, H. K. (ed.), Lipids. CRC Press, Boca Raton .

50. **Walan, A., and E. Kihlstrom**. 1988. Surface charge and hydrophobicity of *Campylobacter jejuni* strains in relation to adhesion to epithelial HT-29 cells. APMIS. **96:** 1089-1096.

51. **Weerkamp, A. H., H. C. van der Mei, and J. W. Slot**. 1987. Relationship of cell surface morphology and composition of *Streptococcus salivarius* K^+ to adherence and hydrophobicity. Infect. Immun. **55:** 438-445.

52. **Weinhold, P. A., M.-E. Rounsifer, and D. A. Feldman**. 1986. The purification and characterization of CTP:phosphorylcholine cytidylyltransferase from rat liver. J. Biol. Chem. **261:** 5104-5110.

*CHAPTER 4

4.1. *CAMPYLOBACTER JEJUNI* PROTEINS INVOLVED IN LIPID HYDROLYSIS

4.1.1. INTRODUCTION

Campylobacter jejuni is a gram-negative organism that is the leading cause of acute gastroenteritis in developed and developing countries. In the United States, *C. jejuni* is reported to be more common than *Salmonella* and *Shigella* combined, with an estimated annual incidence of 2 million cases (6). In developing countries, *C. jejuni* ranks as the third most common cause of childhood diarrhea after enterotoxigenic *E. coli* and rotavirus (33). The illness is usually self-limiting but extra-intestinal complications such as Guillain-Barré syndrome, reactive arthritis, Reiter's syndrome, meningitis, abortions, cholecystitis, bacteremia and urinary tract infections have been reported (6). Although *C. jejuni* infects large numbers of people worldwide, there is a lack of information describing the pathogenic mechanisms of this organism. Proposed virulence factors of *C. jejuni* include adherence, invasiveness, motility, lipopolysaccharide, flagella, pili, and toxin(s). It is necessary to investigate the interactions between the organism and its host in order to be able to determine methods of intervention and prevention of disease.

We recently showed that *C. jejuni* interacts with lipids (37). We speculated that the protein described may be involved in lipid metabolism or lipid hydrolysis. After

---

* The manuscript presented in this section is the same as that submitted to Infection and Immunity (January, 1997) by C. M. Szymanski, M. Haardt, D. A. Rasko, and G. D. Armstrong. Dr. M. Haardt was responsible for repeating the crosslinking experiment with excess PE shown in Figure 4.1. D. A. Rasko was responsible for the lipase chaperone western blot shown in Figure 4.3 and for the sensitivity experiment shown in Figure 4.6.

investigating this interaction further, we identified several *C. jejuni* proteins involved in lipid hydrolysis.

In a recent review, Waite described the ubiquity of lipid hydrolysing enzymes (42). Proteins involved in lipid hydrolysis include the phospholipases which hydrolyse phospholipids and the lipases which hydrolyse triacylglycerols. The phospholipase family can be divided into two groups. The first group, the acyl hydrolases, attack the acyl ester at position 1 of the glycerol backbone (phospholipase A_1, PLA_1), at position 2 (phospholipase A_2 (PLA_2), or at both positions (phospholipase B, PLB). The second group, the phosphodiesterases, hydrolyse the linkage between the phosphate and the glycerol backbone (phospholipase C, PLC), or between the phosphate and the head group (phospholipase D, PLD). Alternatively, the lipases hydrolyse triacylglycerols at one or more of the three carboxyl ester bonds.

Acyl hydrolases are usually cell-associated proteins exhibiting a broad-specificity for substrates (41). PLA_2 enzymes are small proteins with molecular weights of 12-15 kDA (42). PLA_2 activity has been shown to exist in several other pathogenic bacteria, including the cell sonicates of *C. jejuni* (24).

Phosphodiesterases are usually soluble proteins that are often secreted. The main role of PLC may be to maintain phosphate supplies (24, 40). The size of PLC is quite heterogeneous ranging between 18-78.4 kDa (40). Sphingomyelinase C, enzymes which specifically hydrolyse sphingomyelin with the release of ceramide, are generally considered part of the PLC group (40).

Lipases are usually secreted by bacteria (14). These proteins are also heterogeneous in size ranging from 15-76 kDa (14, 35). The physiological role of lipases may be nutritional (27). Several *Pseudomonas* strains contain lipase chaperone genes linked to the lipase structural genes (*B. cepacia*-LimA, *P. aeruginosa*-Act, LipH, LipB, and LimL, *P. glumae*-LipB) (14). *Acinetobacter calcoaceticus* has also recently been shown to contain a lipase helper protein known as LipB (19).

It has been suggested that lipid hydrolysing enzymes may directly damage cells and mucus layers as well as stimulate the production of secondary messengers which may initiate inflammation, apoptosis and premature labor in humans (2, 14, 27, 30, 40, 42). Therefore, these enzymes are potentially important virulence factors. This paper describes the identification of several *C. jejuni* lipid hydrolysing proteins and the possible roles these proteins may play in pathogenesis.

4.1.2. MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *C. jejuni* strain UA580 was kindly provided by D. E. Taylor, Medical Microbiology and Immunology, University of Alberta. *C. jejuni* strains ER1109 and E863 were obtained from the Provincial Laboratory of Northern Alberta. *C. jejuni* strain 81116 was kindly provided by T. J. Trust, Biochemistry and Microbiology, University of Victoria. The organisms were grown as previously described (38).

Membrane Isolation. Bacterial membranes were isolated according to the method of Stenson and Peppler (36). An overnight culture of *C. jejuni* was centrifuged at 16,000 X g for 20 min and the bacteria were washed twice with 10mM HEPES, pH 8.0. The pellets were resuspended in lithium buffer (200 mM lithium chloride, 100 mM lithium acetate, pH 6.0) with 100 μM PMSF at a ratio of 0.5g pellet to 1ml buffer. The sample was then sonicated in a pre-chilled B-220 ultrasonic cleaner (Branson Cleaning Equipment) for 10 min. Cell debris was removed by centrifugation at 12,000 X g for 20 min. This step was repeated to ensure complete removal of all intact cells. The supernatant was then centrifuged at 125,000 X g for 1 hour at 4°C. The resulting supernatant was discarded and the membrane pellets were resuspended in HEPES buffer and recentrifuged at 125,000 X g for 1 hour at 4°C. The pellet was resuspended in 10 mM potassium phosphate buffer (pH

7.4) containing 100 μM Triton X-100 and frozen. Protein concentration was determined by the Pierce BCA protein assay using BSA as the standard.

^{125}I-SASD-Crosslinking. Sulfosuccinimidyl 2-(*p*-azidosalicylamido) ethyl-1-3-dithiopropionate (SASD, Pierce) was incubated with Na^{125}I (1 SASD : 1.5 ^{125}I, molar ratio, Amersham) in a 1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril-coated (IODO-GEN, Pierce) tube in the dark for 2 min. ^{125}I-SASD was then incubated with phosphatidylethanolamine (PE) overnight in a solution containing a proton acceptor, imidazole, (6.667 mg/ml) in dry DMSO (dried over 3Å molecular sieves). The following molar ratios were used to determine the amount of reactants used: 2 SASD : 1 PE and 1 PE : 2 imidazole.

The next day, a 10% (w/w) substituted hydroxyalkoxypropyl derivative of Sephadex G-25 known as Lipidex 1000 (Sigma) was added to the mixture and incubated at 37°C for 1 hr. PE covalently attached to ^{125}I-SASD binds to the Lipidex while unreacted ^{125}I and SASD remains in the supernatant (see Glatz *et al.* (8)). After four washes with phosphate buffered saline, ^{125}I-ASD-PE was eluted with methanol. The solvent was evaporated under nitrogen and the modified lipid was sonicated into 10 mM potassium phosphate buffer (pH 7.4) containing 100 μM Triton X-100. ^{125}I-ASD-PE was then incubated with either whole organisms or membrane preparations in the presence or absence of 1000-fold excess unlabelled PE for 1 hour at room temperature. To covalently attach the modified PE to proteins, the arylazide was activated by ultraviolet light for 10 min. The samples were then analyzed by SDS-PAGE and autoradiography.

Non-denaturing Polyacrylamide Gel Electrophoresis. Native gels were prepared according to the methods of Dulaney and Touster and Koide *et al.* (3, 17). A 6% polyacrylamide gel was prepared with the addition of 0.1% Triton X-100 (w/v) to both the separating and stacking gels. The running buffer was prepared as described by Laemmli with the exception that 0.1% Triton X-100 (w/v) was substituted for SDS in the upper

chamber of the apparatus (23). The sample buffer contained: 1 % Triton X-100 (w/v), 25 % glycerol (v/v), and 0.02% saturated bromophenol blue (v/v) in 1.0 M Tris buffer, pH 6.8. The gel was run at a constant 50 volts and stained with Coomassie blue (45% methanol (v/v), 10% acetic acid (v/v), and 0.2% coomassie brilliant blue R-250 (w/v) in water) for 0.5 hr. Gels were destained (25% methanol (v/v) and 8% acetic acid (v/v) in water) overnight.

^{3}H-PC Binding and Native Gel Electrophoresis. A *C. jejuni* membrane preparation was incubated with ^{3}H-phosphatidylcholine (PC). The product was then analyzed on a non-denaturing gel. The gel was coomassie stained, destained and then soaked in Amplify (Amersham) before drying. Autoradiograms of the gels were then prepared using Hyperfilm MP (Amersham).

Removal of Protein from non-denaturing polyacrylamide gels. Proteins were purified according to the acid extraction procedure described by Kellner (16). Large-scale membrane preparations were analyzed on native gels, stained with Coomassie blue, and destained overnight. The appropriate band was then cut out of each gel. The gel slices were cut into small pieces, approximately 2mm square, and placed into Eppendorf tubes. Gel pieces were covered with water and allowed to stand for 10 minutes. The liquid was then removed and a mixture of acetonitrile/water (1:1, vol/vol) was added for 20 minutes. This step was repeated until the liquid was clear. Then, 70% formic acid (vol/vol) was added and the samples were incubated for 30 min at 37°C. The liquid was collected and a second extraction with formic acid was done. Then, three successive extractions with formic acid/acetonitrile (1:1, vol/vol) were done under the same conditions. The extracts were pooled, evaporated to dryness, and washed in water. The samples were then evaporated to dryness and sonicated into 10 mM potassium phosphate buffer, pH 7.4, with 150 mM KCl and 100 μM Triton X-100.

Preparation for N-terminal Sequencing. The boiled samples from the formic acid extraction procedure were loaded onto a 15% polyacrylamide denaturing gel and run at a

constant 100 volts. Proteins were transferred onto an Immobilon-P (Millipore) membrane at 0.5 A for 20 min in transfer buffer (10mM CAPS, 10% methanol (v/v), pH 11.0). The membrane was washed 3 times with water, stained with Coomassie blue (0.1% w/v coomassie brilliant blue R-250 in 50% methanol (v/v) in water), and destained (50% methanol (v/v), 10% acetic acid (v/v) in water). The membrane was dried and the bands were cut out and submitted to the Alberta Peptide Institute for sequence analysis.

Western Blots. *C. jejuni* membrane proteins and lipase chaperone standards were analysed on a 15% polyacrylamide denaturing gel as described above. LipH and LimL were added at concentrations of 0.2 μg and 9 μg per lane respectively. The LimA lane contained *E. coli* JA221 whole cell lysates. *E. coli* JA221 contains a plasmid from which LimA is temperature inducible (12). The proteins were transferred onto Immobilon-P membranes (Millipore) for 11 hrs at a constant 27 volts in 25 mM phosphate buffer, pH 7.4. The blots were then blocked overnight at 4°C in TBST buffer (20 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) containing 5% (w/v) skim milk. The next day the membrane was incubated with either LipH antibody (1:50,000), LimL antibody (1:250), or LimA antibody (1:2,500) in TBST (or TBST alone) for 1 hr with constant shaking. The membrane was washed 3 times for 10 min with TBST and then incubated with the alkaline phosphatase conjugated secondary antibody in TBST for 2 hrs with constant shaking. After incubation, the membrane was washed as described above and developed with a NBT/BCIP detection system.

Sodium lauryl sarcosinate (Sarkosyl) Treatment. Sarkosyl was used to selectively solubilize inner membrane proteins as described by Filip *et al.* (5). Briefly, membrane preparations were incubated with 2% (w/v) sarkosyl in 30 mM Tris (pH 8.0) for 30 min. at room temperature. The sample was then centrifuged at 48,000 X g for 45 min. at 4°C. The supernatant which contained soluble inner membrane proteins and the pellet which contained insoluble outer membrane proteins were collected for further analysis.

Preparation of cell sonicates. Sonicates were prepared as described by Langton and Cesareo (24). Briefly, the overnight growth of *C. jejuni* from ten confluent blood agar plates was sonicated in 10mM HEPES, pH 8.0 for 5 min. After sonication, the sample was centrifuged at 18,000 X g for 5 min. at room temperature and resuspended in the same buffer.

Sensitivity experiments. Lipid hydrolysis experiments were done using *C. jejuni* whole cell lysates. An overnight culture of *C. jejuni* was centrifuged at 6000 X g for 6 min. and the bacteria were washed three times with PBS. *C. jejuni* whole cell lysates were prepared by shearing with a hand-held homogenizer followed by sonicating for 10 minutes in Laemmli sample buffer. To determine heat sensitivity of the lipid hydrolysing activities, samples maintained at room temperature were compared to samples heated at 60°C or 100°C for 15 minutes. To demonstrate that the lipid hydrolysing activities were due to proteins, the samples were digested with proteinase K, 0.15 mg/ml final concentration, for 30 min at 37°C. Proteinase K alone was also loaded onto the gel to ensure that this protein did not cause a false positive result. Whole cell lysate sensitivity to dithiothreitol, 50 mM final concentration, was also examined.

Egg-yolk overlay experiment. *C. jejuni* fractions were analyzed on a 6% native polyacrylamide gel at a constant 50 volts. The gel was then equilibrated in 30mM Tris, 0.9% saline, pH 7.5 at room temperature for 30 minutes. Egg yolk agarose was prepared by mixing aspirated egg yolk with PBS containing 1 mM $CaCl_2$ (1:1, vol/vol). The mixture was then added to a cooled solution of 1% agarose in the Tris/saline solution at a concentration of 5% (vol/vol). A thin layer of agarose was poured into a glass dish and allowed to solidify. The equilibrated gel was placed on the hardened agarose and the remainder of the agarose was then poured over the gel. The overlay was incubated overnight at 37°C.

Phospholipase C assay. The assay was done as described by Kurioka and Matsuda (22). The reaction mixture consisted of 0.25 M Tris-HCl, pH 7.2 with a final

concentration of 60% (w/v) sorbitol, 10^{-4} M $ZnCl_2$, and 20 mM *p*-nitrophenylphosphorylcholine (Sigma). To each well, 25 µl of sample or water or PLC standard (2 µg/ml final concentration, from *C. perfringens*, Sigma) was mixed with 100 µl of reaction medium. The plate was incubated overnight at 37°C and the absorbance was measured at 405nm. Note: *C. jejuni* membranes were centrifuged at 125,000 X g for 45 min., washed in water and resuspended by sonication into water to eliminate any detergent affect.

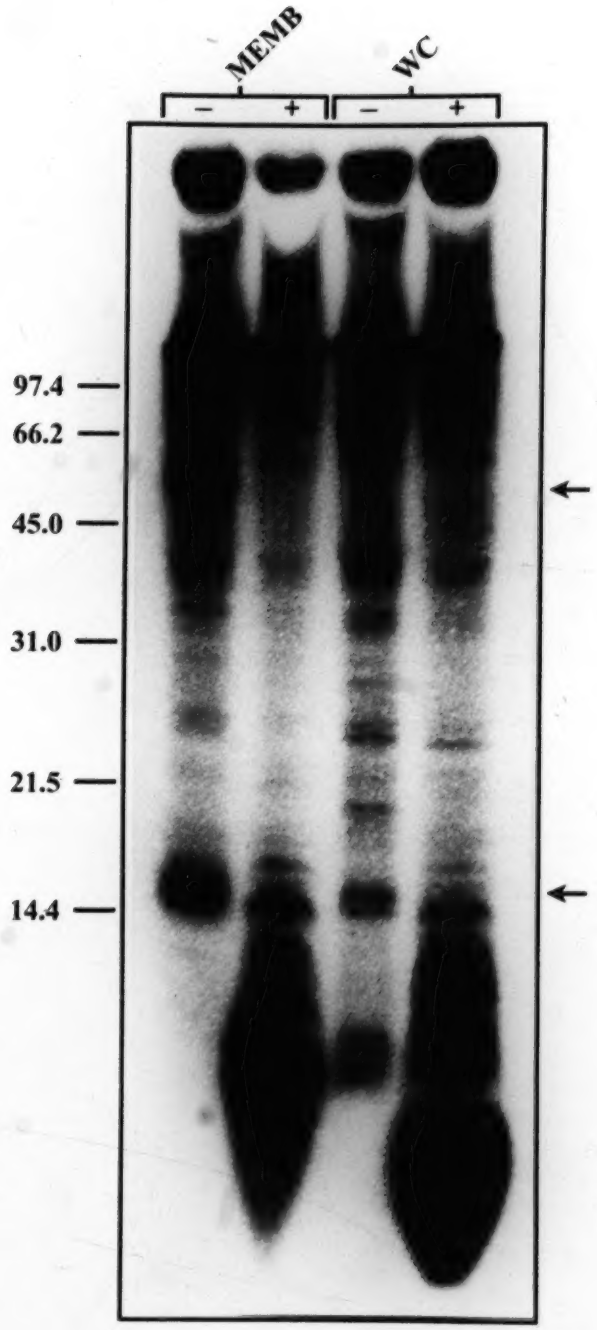
Phospholipase A_2 assay. The assay was done as described by Lôbo de Araújo and Radvanyi (25). The plate was first incubated at room temperature with coating medium (150 mM NaCl, 1% Tween 20 (v/v), and 5 mM sodium phosphate, pH 7.5). The reaction medium contained 4mM sodium cholate, 100 mM NaCl, 10 mM $CaCl_2$, and 0.055 mM phenol red, pH 7.5. At the time of the experiment, 3.5 mM lecithin was added to the reaction medium. To each well, 20 µl of sample or water or PLA_2 standard (0.9 µg/ml final concentration, from *Naja naja* venom, Sigma) and 200 µl reaction medium was added. The plate was incubated overnight at 37°C and the absorbance was measured at 540nm.

Lipase Assay. The assay was done as described by Tirunarayanan and Lundbeck (39). The reaction was adapted for a microtiter plate assay. Each well contained: 5 µl 10% v/v Tween 20 in 50 mM Tris-HCl, pH 7.6; 5 µl 1 M $CaCl_2$ in Tris buffer; 25 µl sample or water or lipase standard (0.17 µg/ml final concentration, from *Pseudomonas* species, Sigma); and 115 µl of 50 mM Tris buffer. The plate was incubated overnight at 37°C and the absorbance was measured at 405nm.

4.1.3. RESULTS

The photoaffinity crosslinking experiments showed that a 14 kDa protein from *C. jejuni* UA580 whole cells and membrane preparations binds to PE (Fig. 4.1). Binding specificity was demonstrated by the ability of excess unlabelled PE to compete for ^{125}I-ASD-PE binding to the protein. Another protein at 55 kDa also selectively bound to PE

Figure 4.1. SDS-PAGE of crosslinking experiment between ^{125}I-SASD-derivatized phosphatidylethanolamine (PE) and *C. jejuni* UA580 whole cells (WC) and membrane preparations (MEMB) with (+) or without (-) excess underivatized PE. The specific labelling of the 55 kDa protein in the membrane preparation (-PE) and its disappearance in the presence of excess unlabelled PE (+PE) is shown by the top arrow. The lower arrow indicates the position of the 14 kDa band (higher band of doublet) in the presence of excess unlabelled PE.


MEMB
WC
–
+
–
+
97.4
66.2
45.0
31.0
21.5
14.4

however, this protein was visible only in the membrane preparation. Several other proteins were labelled non-specifically in this procedure since excess PE did not compete for binding.

C. jejuni membrane preparations incubated with ^{3}H-PC and then analyzed by native gel electrophoresis exhibited a single radioactive band (Fig. 4.2, lane B). The labelled band observed in Fig. 4.2, lane B corresponds to the second of the protein bands shown in the Coomassie stained gel in Fig 4.2, lane A. This second protein band was the same protein band responsible for the precipitating activity demonstrated in the native gel egg yolk overlays (Fig. 4.4 and 4.5). This observation was used as the basis for purifying the 14 kDa protein. *C. jejuni* UA580 membrane preparations were separated by native gel electrophoresis. The second major protein band was excised and eluted by acid extraction. Several proteins were observed by SDS-PAGE (results not shown). Although the 55 kDa protein was not visible by Coomassie staining, the 14 kDa protein was detected. The 14 kDa band was cut out and sequenced.

Sequencing results showed that there were two proteins comigrating at the 14 kDa range. However, since the proteins were present in different molar amounts, it was possible to obtain sequence data for both. The protein at the higher concentration was not homologous to any protein in the GenBank and SwissProt data banks however, the other protein exhibited 62.8% homology to the lipase chaperone proteins of *Pseudomonas* species (Table 4.1). Antisera raised against *Pseudomonas* chaperones LimA and LipH, but not LimL, crossreacted with a 14 kDa protein in western blots of *C. jejuni* membrane preparations (Fig. 4.3). Also, anti-LimL crossreacted with LipH (Fig. 4.3, lane 2). Two *C. jejuni* higher molecular weight proteins appeared to react with the lipase chaperone antibodies. However, these proteins can also be seen in the last panel in this figure in which only the rabbit secondary antibody was used to probe the blot. Therefore, reactivity to these two proteins is not specific. The rabbit secondary antibody did not react with the *C. jejuni* 14 kDa protein (Fig. 4.3, lane 1).

Fig. 4.2. ^{3}H-PC autoradiogram demonstrating the presence of lipid binding activity in *C. jejuni* UA580 membrane preparations. *C jejuni* membrane proteins were incubated with ^{3}H-PC and then separated by native gel electrophoresis. The Coomassie stained gel is shown in Lane A. The autoradiogram is shown in lane B. The hydrophobic nature of the tritiated phospholipid results in slow migration of unbound lipid into the separating gel. However, the second major protein band of the membrane preparation (shown by the arrow) is solely labelled by the tritiated phospholipid.

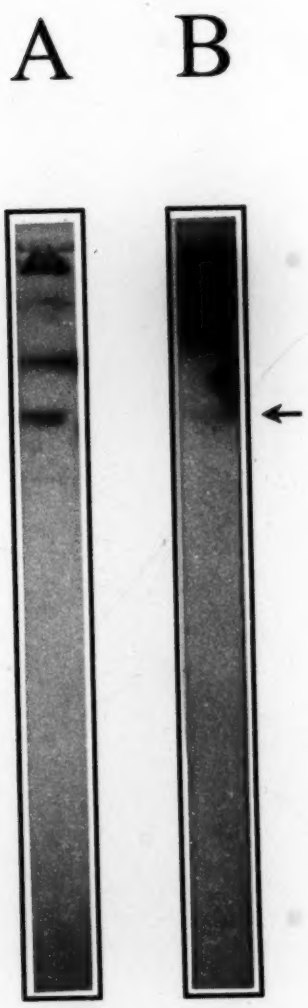

A
B

TABLE 4.1. Molecular weight and amino acid comparisons of *C. jejuni* UA580 identified proteins

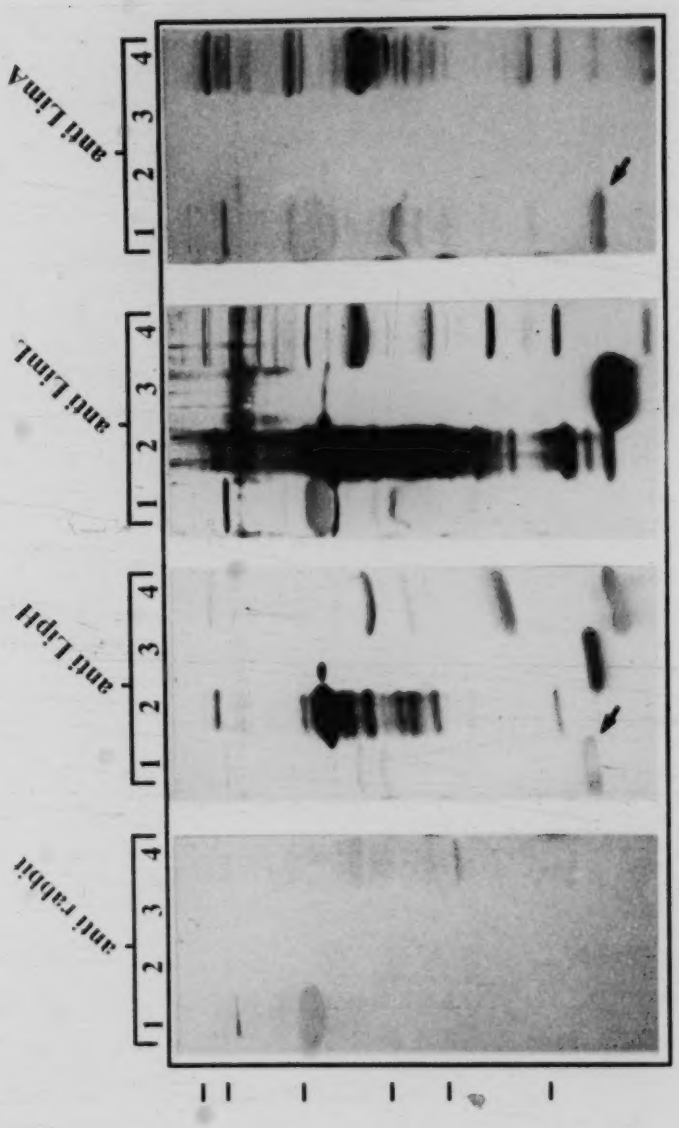
Organism	Protein	MW[a]	Amino Acid Sequence[b,c]	Residues	Reference
C. jejuni	lipase chaperone	14	XXXQGAVLDYKRNL--V--V--LEXS	1-21	This study
P. aeruginosa PAO1	lipase chaperone LipH	33.6	-------IDYKKEL--V--L--LERD	82-94	(7, 44)
Pseudomonas strain 109	lipase chaperone LimL	30-43	-------IDYKKEL--V--L--LERD	140-152	(7, 13, 19)
A. calcoaceticus	lipase chaperone LipB	39	-------LKYREEL--G--N--LKEP	137-149	(19)
B. cepacia	lipase chaperone LimA	33	-------V-WHRYRAYLDALAKL-RD	139-155	(12, 44)
C. jejuni	hydrolase or transporter	14	XXYKEDYRELDSTSGQPAXFD[c]	1-21	This study

[a] Molecular weight in kDa

[b] X represents unidentified amino acid

[c] Identical amino acid matches with the *C. jejuni* sequence are underlined

Figure 4.3. Lipase chaperone crossreactivity experiment. *C. jejuni* UA580 membrane preparations (lane 1), LipH (lane 2), LimL (lane 3), and *E. coli* JA221whole cell lysates with LimA (lane 4) were examined for crossreactivity with anti-LipH, anti-LimL, anti-LimA and secondary antibody (anti-rabbit) antisera. Molecular weight standards appear on the left with the following apparent weights: 21.9, 29.7, 35.1, 50, 97.2, and 142.9 kDa. The arrowheads point to the crossreacting *C. jejuni* 14 kDa protein.


anti Limb
anti Limi
anti Lipii
anti rabbit
1
2
3
4

Egg yolk overlay experiments demonstrated that precipitating activity was present in the whole cell lysates of all four *C. jejuni* strains tested (Fig. 4.4A). However, the activity was not observed in *C. jejuni* concentrated cell-free supernatants which contained 6-fold more protein relative to whole cell lysates (Fig. 4.5A). The activity is present in both sarkosyl insoluble outer membranes and sarkosyl soluble inner membranes (Fig. 4.5A). The shift in inner membrane protein mobility is believed to be due to the presence of sarkosyl in the preparation since we observed protein shifts in native gels by using different detergents (results not shown). Lipid hydrolysing activity was present after incubation at 60°C but not after boiling (Fig. 4.6, lanes 2 and 3, respectively). Activity was also observed after dithiothreitol treatment but not after proteinase K digestion (Fig. 4.6, lanes 6 and 4, respectively).

We then investigated the enzymes involved in the precipitation reaction. In three different microtiter plate assays we looked for the presence of phospholipase C, acyl hydrolase, and lipase activities in *C. jejuni* membrane preparations and cell sonicates. In the phospholipase C assay described by Kurioka and Matsuda, phospholipase C hydrolyses *p*-nitrophenylphosphorylcholine to phosphorylcholine and *p*-nitrophenol in the presence of sorbitol and zinc (22). The release of the chromogen, *p*-nitrophenyl can be measured at 405nm (27, 40). In the acyl hydrolase assay described by Lôbo de Araújo and Radvanyi, acyl hydrolases cleave lecithin with the liberation of fatty acids. Using a pH indicator, the change in the pH of the solution can be measured spectrophotometrically at 540 nm (25). In the lipase assay described by Tirunarayanan and Lundbeck, tween 20 is cleaved by lipase to produce fatty acid and alcohol. In the presence of calcium, an insoluble fatty acid salt is formed giving a precipitate which can be measured turbidimetrically at 405 nm (27).

In the assays described, the *C. jejuni* membrane preparation contained both lipase and phospholipase C activities (Table 4.2). In Table 4.2, significant lipase activity was only observed in the membrane preparation ($p=0.022$) and the lipase control ($p=0.030$). The small amount of lipase activity detected in the soluble fraction was not significantly

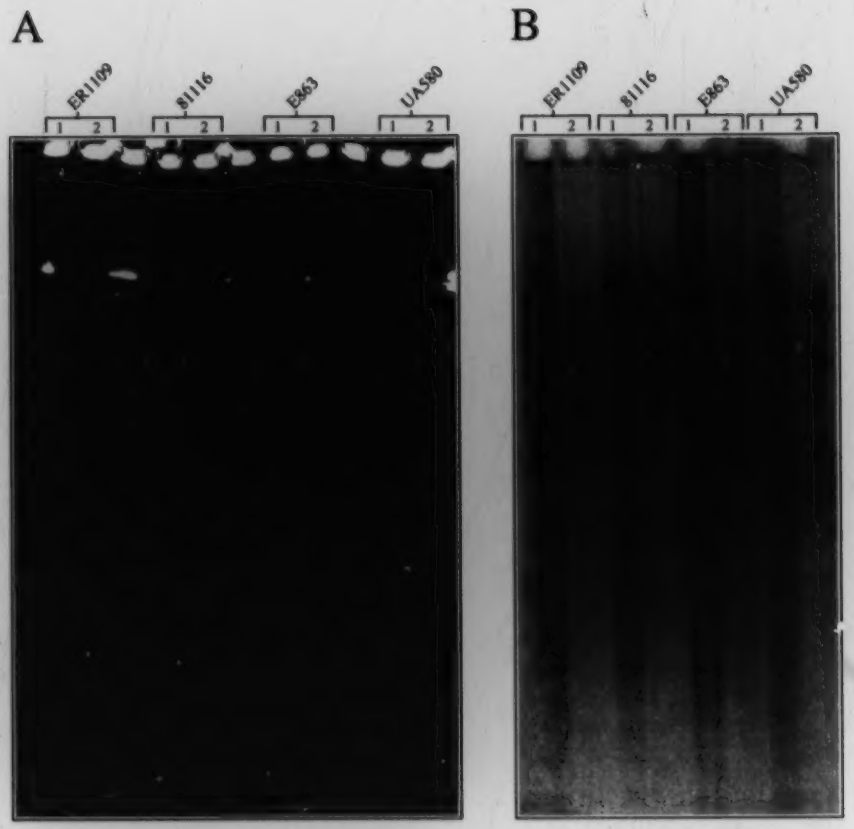


Fig. 4.4. Native egg-yolk overlay gel with *C. jejuni* UA580, *C. jejuni* 81116, *C. jejuni* E863, and *C. jejuni* ER1109 whole cell lysates. Lysates were added to the native gel directly (lane 1) or first diluted three-fold (lane 2). The egg yolk assay is shown in A. The Coomassie stained gel is shown in B. The asterisks show the location of the proteins (B) and their corresponding precipitating acitivities (A).

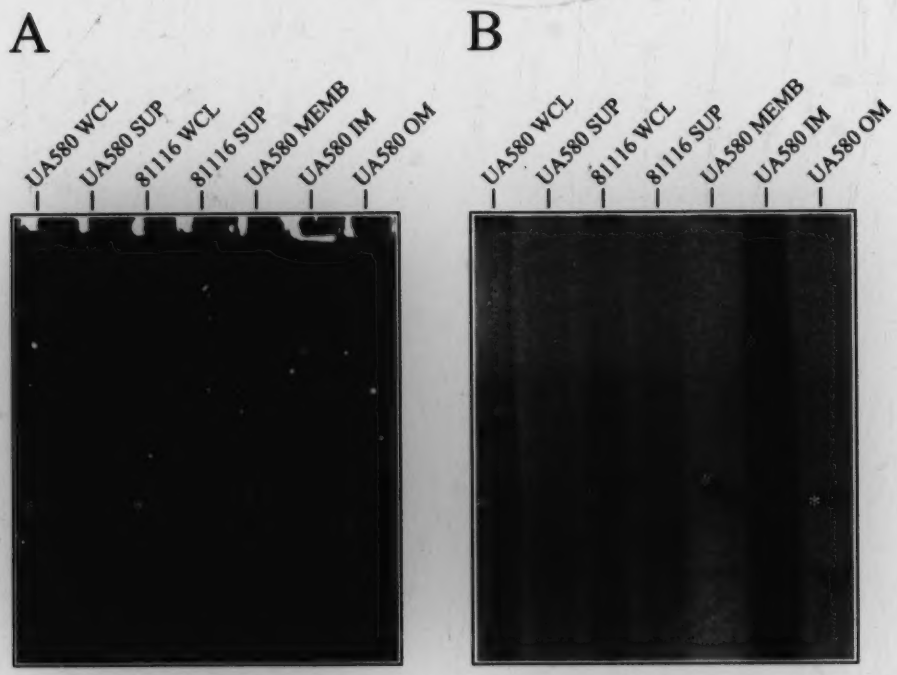


Figure 4.5. Determination of lipid hydrolysing activity in *C. jejuni* UA580 whole cell lysates (WCL), supernatants (SUP), total membranes (MEMB), inner membranes (IM), and outer membranes (OM) by egg-yolk overlay in native gels. Lipid hydrolysing activity in *C. jejuni* 81116 whole cell lysates and supernatants is also shown. The egg yolk assay is shown in A and the Coomassie stained gel is shown in B. The asterisks show the location of the proteins (B) and their corresponding precipitating activities (A).

Figure 4.6. Determination of the sensitivity of C. jejuni UA580 lipid hydrolysing activity to temperature, protein digestion, and disulfide-bond reduction by egg-yolk overlay in native gels. Lane 1 contains untreated C. jejuni whole cell lysates. Lanes 2 and 3 contain the same lysate incubated at 60°C or 100°C respectively. A proteinase K treated lysate is shown in lane 4 while proteinase K alone was added to lane 5. Lane 6 contains a dithiothreitol treated lysate. The egg yolk assay demonstrating precipitating activity in untreated, 60°C-treated, and dithiothreitol-treated lysates is shown. The asterisks show the location of the precipitating activities.

Table 4.2. Determination of lipid hydrolysing activities in *C. jejuni* UA580 membrane preparations and cell sonicates.

Activity	Membrane Units[a]	Cell Sonicate Units	Control Units[b]
Acyl hydrolase	0.056	0.282	0.366
Phospholipase C	0.054 ± 0.023	0.077 ± 0.033	0.047
Lipase	0.168 ± 0.008	0.065 ± 0.050	0.535 ± 0.023

[a]Activity units for the acyl hydrolase assay are expressed as the positive change in the optical density at 540 nm per hour ± SE. Activity units for the PLC and lipase assays are expressed as the change in the optical density at 405 nm per hour ± SE.

[b]Commercially available enzymes were used as controls for the assays (acyl hydrolase: PLA_2; PLC: PLC; and lipase: lipase).

Acyl hydrolase activities were calculated from a single experiment using the same membrane preparation and cell sonicate used in the other microtiter assays. Phospholipase C determinations were calculated from quadruplicate experiments. Phospholipase C control activity, at the concentration described, was determined from a single experiment. Lipase activity was determined from duplicate experiments. Experimental results were analyzed by the Student's t-test where $p < 0.05$ was considered to be statistically significant.

greater than the activity in blank wells (p=0.422). Both the membrane preparation and the soluble fractions contained PLC activities above background levels. The *C. jejuni* cell sonicate and phospholipase A_2 control demonstrated acyl hydrolase activity above background levels (Table 4.2). Very little acyl hydrolase activity was observed in the membrane preparations.

4.1.4. DISCUSSION

Photoaffinity crosslinking results demonstrated that *C. jejuni* proteins at 14 and 55 kDa bound specifically to lipids since excess unlabelled PE was able to compete for ^{125}I-ASD-PE binding to the proteins. The 55 kDa protein was labelled in the membrane preparation but not in whole cells. Although it is possible that the lipid modified crosslinker could pass through the bacterial outer membrane, the movement of phospholipids across the membrane is restricted (40). Since the crosslinker is incubated with bacterial whole cells for only 10 min., it is unlikely that inner membrane proteins are labelled in this procedure. The absence of the labelled 55 kDa protein in whole cells may suggest that the protein is an inner membrane protein. Alternatively, the 55 kDa protein may not be properly exposed to the crosslinker when in the intact bacterial membrane and may only be labelled when present in the membrane preparation. It is also interesting to note that large amounts of radiolabel are present near the gel dye front in lanes containing excess PE. It is suspected that some of the excess PE itself becomes labelled due to micellular interactions between PE and ^{125}I-ASD-PE. Similarily, autolabelling resulting from intersubunit crosslinking in oligomeric proteins has been previously reported (1).

The egg yolk overlay procedure was previously shown to detect lecithinase activity (4, 40, 41). However, Lonon *et al.* argued that egg yolk is composed of lipids, phospholipids, lipoproteins and other components which may act as substrates for several different enzymes (27). They then demonstrated that the egg yolk active enzyme was a lipase rather than a lecithinase. Tirunarayanan and Lundbeck also described the detection

of lipase activity in the egg yolk reaction (39). Alternatively, Lôbo de Araújo *et al.* observed PLA_2 activity using egg yolk as the substrate (26). From these observations it can be concluded that the precipitate formed in the egg yolk reaction could be due to the action of any acylhydrolase (PLA_1, PLA_2, PLB) or phosphodiesterase (PLC, PLD) or lipase. We observed positive egg yolk reactions for PLA_2, PLC, and lipase controls (results not shown). Therefore, a positive egg-yolk reaction suggests that an enzyme has been identified which cleaves fatty acid containing substrates resulting in the deposition of fatty acid salts. However, the type of lipid hydrolysing enzyme involved in the reaction cannot be identified from this assay alone.

Since we identified lipid hydrolysing activity in the egg yolk overlay experiment, we wanted to determine which enzyme(s) was responsible for the activity. From the microtiter plate assays, we observed PLC and lipase activities in *C. jejuni* membrane preparations and PLA_2 and PLC activities in *C. jejuni* cell sonicates. Although acyl hydrolases are generally cell-associated, while phosphodiesterases and lipases are generally secreted, there are some exceptions. Langton and Cesareo found PLA_2 activity in *C. jejuni* and *H. pylori* filtrates (24). Ottlecz *et al.* also reported that *Helicobacter pylori* expresses PLA_1, PLA_2 in both media filtrates and French Press lysates (30). Although PLC activity was present in various *H. pylori* fractions, Weitkamp *et al.* observed the highest PLC activity in membrane fractions (43). The PLC protein of *Ureaplasma urealyticum* is also membrane bound (40). Also, depending on growth conditions, *P. aeruginosa* can exhibit lipase activity that is predominantly associated with the outer membrane (15).

Lipase chaperone proteins (also known as helper or modulator proteins) interact with lipases since the chaperone-lipase complex can be immunoprecipitated using either antibody (14). Lipase chaperones have been shown to modulate lipase folding and to decrease lipase activity within the bacterial cell. The proteins may bind to the lipase to preserve a hydrophobic patch or prevent lipases from aggregating with each other (12). Sequence analysis of the *Acinetobacter* LipB protein suggests that the protein is anchored to

the inner membrane of the cell by its N-terminus with possible activity in the periplasm (19). *Pseuodomonas* LimL and LipH are also believed to be associated with the inner membrane and to be functional in the periplasm (13, 15). Therefore, lipase chaperones may also be involved in lipase translocation.

The N-terminus of one of the *C. jejuni* 14 kDa proteins exhibited 62.8% homology to the lipase chaperone family of *Pseudomonas*. However, amino acid alignment demonstrated that the N-terminus of the 14 kDa *C. jejuni* protein was homologous to more distant regions of the reported chaperone sequences (Table 4.2). Also, the lipase chaperones have larger molecular weights than the 14 kDa *C. jejuni* protein (Table 4.2). These results suggest that the *C. jejuni* 14 kDa protein may be a truncated or functionally modified version of the identified bacterial lipase chaperones. However, our data demonstrate that the *C. jejuni* 14 kDa protein exhibits sequence and antigenic similarity with the lipase chaperone family. Also, the first report of a chaperone in a non-*Pseudomonas* organism, *Acinetobacter,* suggests that similar chaperones may be present in other lipase producing Gram-negative bacteria.

Lipases, especially phospholipases, play an important role in virulence (27). PLC may be responsible for some of the skin damage caused by *P. aeruginosa* in burn patients (40). The enzyme has also been suggested to be responsible for the cytopathology of *Pseudomonas* lung infections (40). *Listeria monocytogenes* expresses two different PLC enzymes: the broad range PLC and the PI-specific PLC. A double mutant in these enzymes demonstrated 500-fold less virulence in mice compared to the parental strain (34). The PLC of *Clostridium perfringens*, known as alpha toxin, is believed to play a key role in the production of gas gangrene by activitating a secondary messenger cascade (40).

Lipases have also been suggested to play roles in *Propionibacterium acnes* acne formation and colonization/persistence as well as in *Staphylococcus epidermidis* and *Staphylococcus aureus* skin colonization (14). Lipases have been proposed to act synergistically with PLC (14). Bacterial PLA_2 has been implicated in playing a role in

initiating premature labor (2). Langton and Cesareo suggested that PLA_2 enzymes may hydrolyse membrane lipids and initiate inflammation (24).

Helicobacter pylori, has been reported to express phospholipase and lipase activity (30). Ottlecz *et al*. suggested that *H. pylori* colonization may disrupt the stomach barrier by hydrolysing the phospholipids lining the cell membranes or in the mucus layer (30). Since *C. jejuni* also swims through and colonizes the intestinal mucosa, lipid hydrolysing enzymes would be beneficial to the organism and possibly detrimental to the host during these processes.

Also, sphingomyelin-hydrolysing PLC's are suggested to be involved in the induction of apoptosis through the elevation of intracellular ceramide levels. *L. monocytogenes* infected cells showed increased levels of diacylglycerol and ceramide (34). It has been shown that *L. monocytogenes* induces apoptosis in infected hepatocytes however, a link between ceramide production and apoptosis has not been established (31). Also, *H. pylori*, another organism which produces PLC, has recently been shown to induce epithelial apoptosis *in vivo* (29). Cell monolayers infected with *C. jejuni* have been reported to show cell rounding, loss of adherence, and death after 24-48 hours (20, 21). *C. jejuni* infection in infant monkeys also showed damaged epithelial cells which exhibited premature apoptosis (32). It would be interesting to investigate the phospholipid specificity of the *C. jejuni* PLC.

We describe the first report of PLC and lipase activities and the identification of a potential lipase chaperone in *Campylobacter jejuni*. We also confirm the presence of PLA_2 in *C. jejuni* cell sonicates which was previously described by Langton and Cesareo (24). We suspect that these enzymes may play a metabolic role possibly by aquiring phosphate and fatty acids. The lipid hydrolases may also be involved in the hydrolysis of cell membrane lipids or mucus lipids, in inflammation, in apoptosis, or in another signal transduction pathway. We are continuing to characterize the lipid hydrolases described in this paper in order to elucidate their potential roles in pathogenesis.

4.2. RELATED WORK

This section shows further supporting data that two lipid hydrolysing activities are found in *C. jejuni* membranes as well as presenting arguments against the possibility that the enzymes could be esterases. Also, further analysis of the unmatched 14 kDa sequence is done and sequencing results of a 55 kDa protein are presented.

Egg yolk overlay experiments with native gels containing sodium dodecyl sulfate demonstrated two *C. jejuni* lipid hydrolysing activities at 14-20 and 45-65 kDa (Figure 4.7). This observation supports the microtiter plate assay results which demonstrated two lipid hydrolysing activities in the membrane preparation (PLC and lipase). In egg-yolk overlay experiments with gels containing SDS, gel electrophoresis was performed according to the method of Laemmli (23). However, the sample buffer was prepared without dithiothreitol and the samples were not heated prior to analysis.

In addition to phospholipase and lipase activity, the precipitating activity observed in the egg yolk overlay may be due to an esterase. Esterases and lipases are both carboxylic ester hydrolases (18). However, esterases hydrolyse water-soluble or emulsified esters with relatively short fatty acid chains whereas lipases (triacylglycerol acyl hydrolases) preferentially hydrolyse emulsified substrates with long-chain fatty acids. *Acinetobacter calcoaceticus* produces several lipolytic enzymes with overlapping specificities: LipA, EstA, tributyrin esterase, and Tween esterase (19). However, egg yolk and olive oil are described as indicators for lipase activity while tributyrin is used as the indicator for esterase activity (19). Also, Dulaney and Touster demonstrated that SDS inhibits esterase activity while we observed two zones of precipitate using SDS in the egg yolk overlay procedure (3). These observations suggest that the precipitating activity is not due to esterases.

Figure 4.8. demonstrates the preparatory gel and transfer membrane used in the preparation for N-terminal sequencing. The second major protein band on native gels was demonstrated to contain lipid hydrolysing activity. Interestingly several proteins were

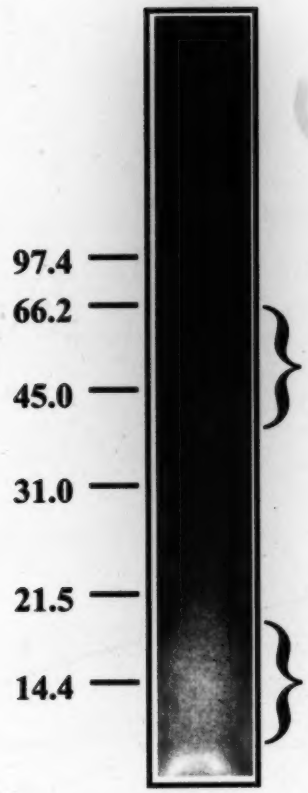


Figure. 4.7. Egg-yolk overlay of a *C. jejuni* UA580 membrane preparation separated by gel electrophoresis in the presence of SDS. Two zones of activity, shown by the brackets, are observed when SDS is present in the sample and running buffers. The bright areas above and below the precipitates are artifacts due to photography (gel edges along the sides, wells or between the separating and stacking gels may reflect light depending on the angle of the light during photography).

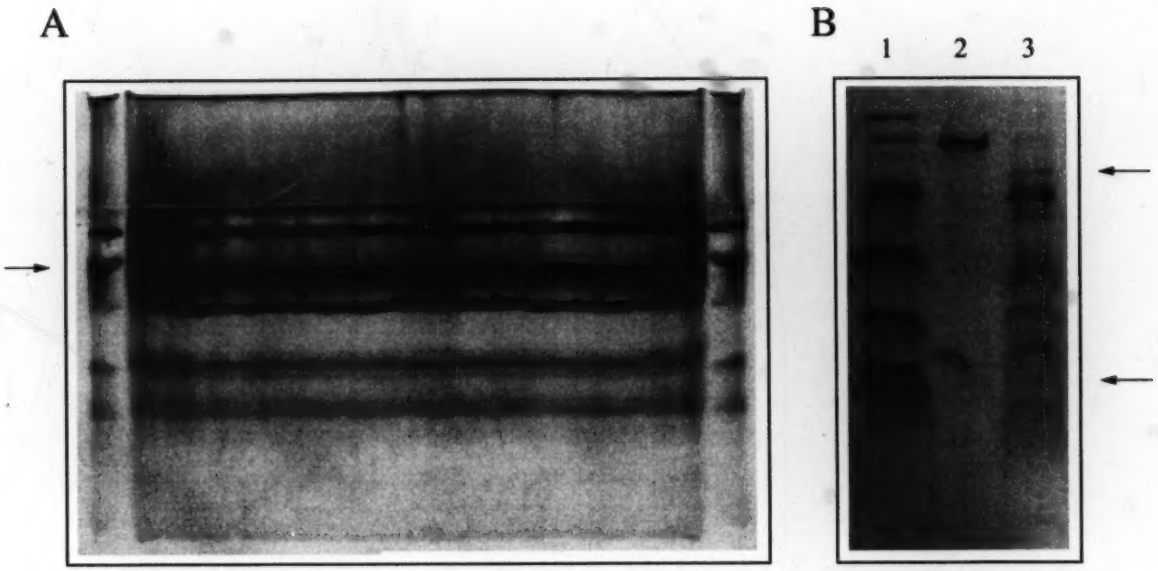


Figure 4.8. Preparation for N-terminal sequencing. (A) shows the native preparatory gel from which the second major protein band (arrow) was excised for acid extraction. (B) shows the blot from which protein bands were cut and submitted for N-terminal sequencing. Lane 1 contains low molecular weight markers (Bio-Rad) with the following molecular weights: 14.3, 21.5, 30, 46, 66, 97.4 kDa. Lane 2 contains the bovine serum albumin standard used for sequencing. Lane 3 contains the *C. jejuni* membrane proteins that were acid extracted from the gel shown in A. The arrows indicate the two bands (14 and 55 kDa) that were cut out for sequencing.

separated from this band by SDS-PAGE. The major band in Figure 4.8.B. is believed to be the *C. jejuni* major outer membrane protein based on its molecular weight and relative concentration. The results of N-terminal sequencing of the 14 kDa bands have already been described. Since one of the 14 kDa proteins did not match any proteins in the data banks, the protein was compared to identified *C. jejuni* proteins with similar molecular weights. The region upstream from the *C. jejuni flhA* gene contains an open reading frame which encodes a 15,188 kDa protein (see section 1.2.6. for a description of *flhA*, (28)). This open reading frame, which overlaps with *flhA* by 14 nucleotides, is homologous with the putative response regulator protein and with the adenylosuccinate synthetase encoding gene *purA*. However, the open reading frame shows no homolgy with the 14 kDa protein sequenced. Also, there is no homology between the 14 kDa protein sequenced and the 18 kDa protein known as Omp18 (see section 1.2.12.).

N-terminal sequence analysis of the 55 kDa protein demonstrated a match with the 56 kDa *C. jejuni* trigger factor protein which was identified by Griffiths *et al.* (Table 4.3, (10)). They demonstrated that the *C. jejuni* trigger factor shows 31% identity with the amino acid sequence of the *E. coli* trigger factor protein using the programs FASTA and BLASTX. The protein is believed to act as a chaperone that is somehow involved in cell division since trigger factor depleted or overproducing *E coli* cells show incomplete septation (11). Griffiths previously demonstrated that Campylobacters become more filamentous later in their growth cycle (see section 1.1.1., (9)). The overproduction *of C. jejuni* trigger factor in *E. coli* also resulted in cell elongation (10). However, *E. coli* trigger factor antiserum did not react with the *C. jejuni* protein in western blotting (10). We as well as Griffiths *et al.* observed that the *C. jejuni* trigger factor is immunogenic during infection (10). The gene has two potential start codons responsible for two potential protein products, one at 56 kDa and one at 52 kDa. The protein that was isolated was similar to the 56 kDa protein (10). Griffiths *et al.* speculate that the 52 kDa protein is a similar protein, less 22 amino acids, although they have not shown that it may be a

TABLE 4.3. IDENTIFICATION AND COMPARISON OF TRIGGER FACTOR N-TERMINAL AMINO ACID SEQUENCES

Organism	Protein	Amino Acid Sequence[a,b]	Residues	Reference
C. jejuni	trigger factor Tig	XEVKAKOLDVVNATASVKIPD	1-21	Chapter 4
C. jejuni	reported trigger factor	MEVKAKOLDSVNATASVKIPS	1-21	(10)
E. coli	trigger factor Tig	MQVSVETTQGLGRRVTITIAA	1-21	(10)

[a] X represents unidentified amino acid

[b] Identical amino acid matches with the *C. jejuni* sequence are underlined

posttranslationally modified version of the 56 kDa protein. The gene contains an unusual σ54-like promoter that is also used by the *flaB* gene of Campylobacters (see section 1.2.4.). Initial data by Griffiths *et al.* suggest that the regulation of the gene complex, with several start sites, is growth-related (10). Interestingly, downstream from *tig* is another open reading frame encoding a protein which is homologous to the ATP-dependent ClpP protease of *E. coli*. From initial data Griffiths *et al.* speculate that the two proteins are cotranscribed. When we first compared the N-terminus of our 55 kDa protein with the BLITZ program, several nonsignificant matches with phospholipase A_2 were observed. It may be possible that products of *tig* and *clpP* are directly involved in septation by hydrolysing lipids and proteolitically cleaving proteins which separate the two daughter cells. Alternatively, the 55 kDa lipid binding protein that we expected to sequence may not have been in high enough concentration to detect and a greater purification may be necessary to obtain enough of this protein for N-terminal sequence analysis.

A summary of the proteins and activities identified in this chapter is shown in Table 4.4.

Table 4.4. SUMMARY OF PROTEINS / ACTIVITIES IDENTIFIED

Protein	MW (kDa)	Location	Function	Determination of function	N-terminal sequence
Lipid hydrolase or transport protein	14[a]	membrane	lipid binding	^{125}I-crosslinking to PE ^{3}H-PC binding[b] Lipidex 1000 assay with PC[c]	no homologs
PLC or Lipase	14-20	membrane	lipid hydrolysis	Microtiter plate assay Egg-yolk overlay with SDS	not sequenced
Lipase chaperone	14	membrane	foldase / chaperone	Antibody crossreactivity Sequence analysis	homology to LimL/LipH
Lipid hydrolase or transport protein	55[a]	membrane	lipid binding	^{125}I-crosslinking to PE ^{3}H-PC binding[b]	not sequenced
PLC or Lipase	45-65	membrane	lipid hydrolysis	Microtiter plate assay Egg-yolk overlay with SDS	not sequenced
Trigger factor	56	membrane	cell division	Sequence analysis	homology to Tig
PLA_2	unknown	cell sonicate	lipid hydrolysis	Microtiter plate assay	not sequenced

[a]Since both the 14 and 55 kDa proteins fall within the molecular weight ranges determined to cause lipid hydrolysis, it is possible that one or both of these proteins may have PLC activity

[b]It was not demonstrated that the 55 kDa protein migrated in the second major protein band of native gels or that the 55 kDa protein binds to PC. However, since both the 14 and 55 kDa proteins bind to PE, it is possible that the 55 kDa protein was also involved in ^{3}H-PC binding

[c]The Lipidex 1000 assay with PC will be described in Chapter 5

4.5. REFERENCES

1. **Armstrong, G. D., C. G. Clark, and L. D. Heerze**. 1994. The 70-kilodalton pertussis toxin-binding protein in Jurkat cells. Infect. Immun. **62:** 2236-2243.

2. **Bejar, R., V. Curbelo, C. Davis, and L. Gluck**. 1981. Premature labor. II. bacterial sources of phospholipase. Obstet. Gynecol. **57:** 479-482.

3. **Dulaney, J. T., and O. Touster**. 1970. The solubilization and gel electrophoresis of membrane enzymes by use of detergents. Biochim. Biophys. Acta. **196:** 29-34.

4. **Esselmann, M. T., and P. V. Liu**. 1960. Lecithinase production by gram-negative bacteria. J. Bacteriol. **81:** 939-945.

5. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart**. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115:** 717-722.

6. **Garcia, M. M.** 1990. *Campylobacter*: "the next *Salmonella*". Safety watch - Agriculture Canada. **15:** 1-2.

7. **Gilbert, E. J.** 1993. *Pseudomonas* lipases: biochemical properties and molecular cloning. Enzyme Microb. Technol. **15:** 634-645.

8. **Glatz, J. F. C., and J. H. Veerkamp**. 1983. A radiochemical procedure for the assay of fatty acid binding by proteins. Anal. Biochem. **132:** 89-95.

9. **Griffiths, P. L.** 1993. Morphological changes of *Campylobacter jejuni* growing in liquid culture. Lett. Appl. Microbiol. **17:** 152-155.

10. **Griffiths, P. L., R. W. A. Park, and I. F. Connerton**. 1995. The gene for *Campylobacter* trigger factor: evidence for multiple transcription start sites and protein products. Microbiol. **141:** 1359-1367.

11. **Guthrie, B., and W. Wickner**. 1990. Trigger factor depletion or overproduction causes defective cell division but does not block protein export. J. Bacteriol. **172:** 5555-5562.

12. **Hobson, A. H., C. M. Buckley, J. L. Aamand, S. T. Jørgensen, B. Diderichsen, and D. J. McConnell**. 1993. Activation of bacterial lipase by its chaperone. Proc. Natl. Acad. Sci. USA. **90:** 5682-5686.

13. **Ihara, F., I. Okamoto, K. Akao, T. Nihira, and Y. Yamada**. 1995. Lipase modulator protein (LimL) of *Pseudomonas* sp. strain 109. J. Bact. **177:** 1254-1258.

14. **Jaeger, K.-E., S. Ransac, B. W. Dijkstra, C. Colson, M. van Heuvel, and O. Misset**. 1994. Bacterial lipases. FEMS Microbiol. Rev. **15:** 29-63.

15. **Jaeger, K.-E., B. Schneidinger, K. Liebeton, D. Haas, M. T. Reetz, S. Philippou, G. Gerritse, S. Ransac, and B. W. Dijkstra**. 1996. Lipase of *Pseudomonas aeruginosa*: molecular biology and biotechnological application, p. 319-330. *In* Nakazawa, T. (ed.), Molecular biology of *Pseudomonads*. American Society for Microbiology, Washington, D. C. .

16. **Kellner, R.** (1995, Fragmentation of proteins within a polyacrylamide matrix. Biochemica, p. 19-21.

17. **Koide, M., M. Fukuda, K. Ohbu, Y. Watanabe, Y. Hayashi, and T. Takagi**. 1987. Polyacrylamide gel eletrophoresis of several proteins in the presence of sodium oligooxyethylene dodecyl ether sulfates or a commercially available analog. Anal. Biochem. **164:** 150-155.

18. **Kok, R. G., V. M. Christoffels, B. Vosman, and K. J. Hellingwerf**. 1993. Growth-phase-dependent expression of the lipolytic system of *Acinetobacter calcoaceticus* BD413: cloning of a gene encoding one of the esterases. J. Gen. Microbiol. **139:** 2329-2342.

19. **Kok, R. G., J. J. van Thor, I. M. Nugteren-Roodzant, B. Vosman, and K. J. Hellingwerf**. 1995. Characterization of lipase-deficient mutants of *Acinetobacter calcoaceticus* BD413: identification of a periplasmic lipase chaperone essential for the production of extracellular lipase. J. Bacteriol. **177:** 3295-3307.

20. **Konkel, M. E., S. F. Hayes, L. A. Joens, and W. Cieplak Jr.** 1992a. Characteristics of the internalization and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures. Microbial Pathog. **13:** 357-370.

21. **Konkel, M. E., and L. A. Joens**. 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect. Immun. **57:** 2984-2990.

22. **Kurioka, S., and M. Matsuda**. 1976. Phospholipase C assay using *p*-nitrophenylphosphorylcholine together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. Anal. Biochem. **75:** 281-289.

23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227:** 680-685.

24. **Langton, S. R., and S. D. Cesareo**. 1992. *Helicobacter pylori* associated phospholipase A_2 activity: a factor in peptic ulcer production? J. Clin. Pathol. **45:** 221-224.

25. **Lôbo de Araújo, A., and F. Radvanyi**. 1987. Determination of phospholipase A_2 activity by a colorimetric assay using a pH indicator. Toxicon. **25:** 1181-1188.

26. **Lôbo de Araújo, A., F. Radvanyi, and C. Bon**. 1994. Purification of an acidic phospholipase A_2 from *Bothrops lanceolatus* (fer de lance) venom: molecular and enzymatic properties. Toxicon. **32:** 1069-1081.

27. **Lonon, M. K., D. E. Woods, and D. C. Straus**. 1988. Production of lipase by clinical isolates of *Pseudomonas cepacia*. J. Clin. Microbiol. **26:** 979-984.

28. **Miller, S., E. C. Pesci, and C. L. Pickett**. 1994. Genetic organization of the region upstream from the *Campylobacter jejuni* flagellar gene *flhA*. Gene. **146:** 31-38.

29. **Moss, S. F., J. Calam, B. Agarwal, S. Wang, and P. R. Holt**. 1996. Induction of gastric epithelial apoptosis by *Helicobacter pylori*. Gut. **38:** 498-501.

30. **Ottlecz, A., J. J. Romero, S. L. Hazell, D. Y. Graham, and L. M. Lichtenberger**. 1993. Phospholipase activity of *Helicobacter pylori* and its inhibition by bismuth salts. Dig. Dis. Sci. **38:** 2071-2080.

31. **Rogers, H. W., M. P. Callery, B. Deck, and E. R. Unanue**. 1996. *Listeria monocytogenes* induces apoptosis of infected hepatocytes. J. Immunol. **156:** 679-684.

32. **Russell, R. G., M. O'Donnoghue, J. D. C. Blake, J. Zulty, and L. J. DeTolla**. 1993. Early colonic damage and invasion of *Campylobacter jejuni* in experimentally challenged infant *Macaca mulatta*. J. Infect. Dis. **168:** 210-215.

33. **Siddique, A. B., and S. Q. Akhtar**. 1991. Study on the pathogenicity of *Campylobacter jejuni* by modifying the medium. J. Trop. Med. Hyg. **94:** 175-179.

34. **Smith, G. A., H. Marquis, S. Jones, N. C. Johnston, D. A. Portnoy, and H. Goldfine**. 1995. The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. Infect. Immun. **63:** 4231-4237.

35. **Soberón-Chávez, G., and B. Palmeros**. 1994. *Pseudomonas* lipases: molecular genetics and potential industrial applications. Crit. Rev. Microbiol. **20:** 95-105.

36. **Stenson, T. H., and M. S. Peppler**. 1995. Identification of two bvg-repressed surface proteins of *Bordetella pertussis*. Infect. Immun. **63:** 3780-3789.

37. **Szymanski, C. M., and G. D. Armstrong**. 1996. Interactions between *Campylobacter jejuni* and lipids. Infect. Immun. **64:** 3467-3474.

38. **Szymanski, C. M., M. King, M. Haardt, and G. D. Armstrong**. 1995. *Campylobacter jejuni* motility and invasion of Caco-2 cells. Infect. Immun. **63:** 4295-4300.

39. **Tirunarayanan, M. O., and H. Lundbeck**. 1968. Investigations on the enzymes and toxins of *Staphylococci* - assay of lipase using tween as the substrate. Acta. Path. et Microbiol. Scandinav. **72:** 263-276.

40. **Titball, R. W.** 1993. Bacterial phospholipases C. Microbiol. Rev. **57:** 347-366.

41. **Waite, M.** 1987. The phospholipases. p. *In* Handbook of lipid research. Plenum Press, New York .

42. **Waite, M.** 1990. Phospholipases, enzymes that share a substrate class, p. 1-22. *In* Mukherjee, A. B. (ed.), Biochemistry, molecular biology, and physiology of phospholipase A_2 and its regulatory factors. Plenum Press, New York .

43. **Weitkamp, J. H., G. I. Perez-Perez, G. Bode, P. Malfertheiner, and M. J. Blaser**. 1993. Identification and characterization of *Helicobacter pylori* phospholipase C activity. Int. J. Med. Microbiol., Virol., Parasitol., Infect. Dis. **280:** 11-27.

44. **Wohlfarth, S., C. Hoesche, C. Strunk, and U. K. Winkler**. 1992. Molecular genetics of the extracellular lipase of *Pseudomonas aeruginosa* PAO1. J. Gen. Microbiol. **138:** 1325-1335.

CHAPTER 5

GUILLAIN-BARRÉ SYNDROME AND *CAMPYLOBACTER JEJUNI* INFECTION

5.1. INTRODUCTION

The annual incidence of Guillain-Barré Syndrome (GBS) in the developed world is 1-2 cases per 100,000 (26). An acute infectious illness precedes GBS in up to 70% of patients (13). Approximately 20-40% of patients with GBS have experienced a *C. jejuni* infection 1-3 weeks prior to onset of clinical symptoms (27). The annual incidence of GBS in the USA is estimated at 1.7 per 100,000 suggesting that GBS is a relatively rare disease (26). It can be estimated that 1 in every 2000-5000 cases of *C. jejuni* is followed by GBS (26). A variant of GBS, Miller Fisher syndrome, is also linked to *C. jejuni* infection (8).

So far, no relationship between the severity of *C. jejuni* symptoms and the development of GBS has been reported; even asymptomatic *C. jejuni* infections may trigger GBS (27). However, there are reports demonstrating that GBS patients with preceding *C. jejuni* infection were more likely to have acute symptoms and to have greater disability after one year (30, 32). The *C. jejuni* factor involved in triggering GBS has not been conclusively identified. However, in the USA and Japan, 30-80% of *C. jejuni* isolates from GBS patients belong to Penner serotype O:19 (16, 27). Since the incidence of this particular serotype is low, there appears to be a significant association between this serotype and GBS. In one study, all three *C. jejuni* isolates from patients with GBS belonged to Penner serotype O:19 and all contained LPS with ganglioside-like epitopes (46). Also anti-GM1 antibodies are frequently seen in sera from patients with GBS (46). Since other *C. jejuni* serotypes which contained different terminal ganglioside-like structures were isolated from GBS patients, Yuki *et al.* speculated that infection with *C. jejuni* isolates containing any ganglioside structure on their LPS may lead to the development of GBS (46). Mishu-Allos and Blaser suggested that antibodies generated

against cellular structures such as the O:19 LPS, glycolipids or myelin-like proteins may be the cause of the association (27).

We recently showed that *Campylobacter jejuni* interacts with lipids (38). A *C. jejuni* protein involved in this interaction may belong to the fatty acid binding protein family known as FABP or to the non-specific lipid transfer protein family known as nsL-TP. Interestingly, the binding characteristics and molecular weight of the *C. jejuni* protein (14kDa) are similar to those reported for the proteins belonging to the FABP family. This was particularly intriguing to us because a well known protein of the peripheral nervous system, myelin P2 protein, also belongs to this family. Also, myelin P2 and specific peptides of myelin P2 induce experimental allergic neuritis (EAN), the rat model for human GBS. Antibodies against myelin P2 have occasionally been detected in sera from GBS patients. We therefore speculated that *C. jejuni* may possess a fatty-acid-like binding protein that may share some homology with myelin P2 and that autoreactive antibodies to this protein may cause the demyelinating disorder known as GBS. The motivation for the following studies was to determine whether the *C. jejuni* 14 kDa protein may be linked to the induction of GBS.

We examined myelin P2 and nsL-TP antibody reactivity to *C. jejuni* whole cell lysates. *C. jejuni* membrane preparations were analyzed in the Lipidex 1000 assay commonly used to examine binding specificities of FABP's. Based on these observations we wanted to determine whether the 14 kDa protein is recognized by convalescent sera from patients infected with *Campylobacter* as well as in sera from patients with GBS with or without previous *Campylobacter* infection. We also wanted to determine whether the human sera reacted with another member of the fatty acid binding protein family, adipocyte lipid binding protein (ALBP). Finally, we looked for antibody crossreactivity with several ganglioside structures that have been implicated in initiating GBS.

5.2. MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *C. jejuni* strain UA580 was kindly provided by Dr. D. E. Taylor, Medical Microbiology and Immunology, University of Alberta. The organisms were stored in brain heart infusion broth (BHI, Difco Laboratories) with 10% glycerol (BDH) at -70°C. Cultures were plated on blood agar plates (Triage Microbiological Systems) for 24 hours. The organisms were then inoculated into BHI broth and incubated overnight at 37°C. They were grown under microaerophilic conditions (i.e., 10% CO_2, 5% O_2, 85% N_2) with constant shaking. After centrifugation at 6000 X g for 6 min., the bacteria were resuspended in BHI broth and used in further experiments. Cultures were concentrated and the pellet was washed three times with PBS. *C. jejuni* whole cell lysates were prepared by shearing with a hand-held homogenizer followed by sonicating for 10 minutes in Laemmli sample buffer.

Lipidex 1000 Assay. A modified version of the Lipidex 1000 assay described by Glatz was used (17). Briefly, Lipidex-1000 was first washed with 10 volumes of 10mM potassium phosphate buffer, pH 7.4, and stored as a 50% (v/v) suspension in the same buffer at 4°C. For the assay, 1 nmol of phosphatidylcholine (PC) in chloroform/methanol (2:1, vol/vol) was dried under nitrogen and sonicated into 10 mM potassium phosphate buffer, pH 7.4, containing 100 µM Triton X-100. The *C. jejuni* membrane preparation (see Chapter 4) was incubated with PC for 30 min. in a 37°C water bath. Lipidex 1000 was added to the sample and incubated on an end-over-end rotator for 1 hour in a 37°C incubator. The sample was centrifuged at 3000 X g for 4 min. and the supernatant removed. Cold buffer was added and the sample was incubated for an additional 1 hour on ice. After incubation, the mixture was centrifuged at 3000 X g for 4 min. and the released protein was collected in the supernatant.

Serum Samples. Ten *Campylobacter* positive sera: 4, 8, 13, 24, 36, 39, 42, 48, 53, and 67; five *Campylobacter* positive sera associated with GBS: 1, 9, 21, 55, and 66; and one *Campylobacter* negative serum with GBS symptoms: 716; were kindly provided by Dr. W. Johnson, Laboratory Center for Disease Control, Ottawa. Also included in the

study were sera from 1 patient with GBS-like Miller Fisher Syndrome without any previously known *C. jejuni* infection (MF), a volunteer that had a previously diagnosed *Campylobacter* infection (Prev), and one volunteer without any prior diagnosis of *Campylobacter* infection (Neg).

SDS-Polyacrylamide Gel Electrophoresis. A 15% denaturing gel was prepared according to the method described by Laemmli (20). Boiled *C. jejuni* whole cell lysates were then added and the gel was run at a constant 100 volts. Once electrophoresis was complete, the gel was rinsed in transfer buffer (10mM CAPS, 10% methanol (v/v), pH 11.0).

Western Blots. Proteins were transfered onto Immobilon-P membranes (Millipore) for 11 hrs at a constant 27 volts in 25 mM phosphate buffer, pH 7.4. The blots were then blocked overnight at 4°C in TBST buffer (20 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) containing 5% (w/v) skim milk. The next day the membrane was incubated with either human antisera (1:1000), myelin P2 antibody (1:5000), or nsL-TP (1:2000) in TBST for 1 hr with constant shaking. Myelin P2 antibodies were kindly donated by Dr. B. D. Trapp while nsL-TP antibodies were kindly donated by Dr. K. W. A. Wirtz. The membrane was washed 3 times for 10 min with TBST and then incubated with the alkaline phosphatase conjugated secondary antibody in TBST for 2 hrs with constant shaking. After incubation, the membrane was washed as described above and developed with a NBT/BCIP detection system.

Detection of Lipid Binding by TLC. Binding of human antisera to lipids was evaluated by TLC overlay according to the procedure of Lingwood *et al* (22, 23). Briefly, lipid extracts were separated on silica coated plastic sheets (Polygram SIL-G, Macherey-Nagel) in chloroform/methanol/water (65:25:4, vol/vol/vol). The TLC overlay was blocked with 3% gelatin (w/v, Difco Laboratories) in PBS at 37°C for 2 hours. The plates were then washed and incubated with human antiserum overnight at 4°C. After washing with PBS, the overlays were incubated with alkaline phosphatase-conjugated anti-human

IgG antibody for 1 hour at room temperature, washed with PBS, and developed with a NBT/BCIP detection system.

5.3. RESULTS

Analysis of myelin P2 and nsL-TP antibody crossreactivity with the *C. jejuni* 14 kDa protein. Antibodies against the myelin P2 and the nonspecific lipid transfer proteins were reacted with proteins from *C. jejuni* whole cell lysates (Figure 5.1 arrow). The 14 kDa protein reacted with antibodies against myelin P2. However the protein did not react with antibodies against nsL-TP. These results suggest that the 14 kDa protein may share some antigenic homology with myelin P2. However, other *C. jejuni* proteins also reacted with myelin P2 antibody.

Lipidex 1000 assay. Lipidex 1000 (Sigma) is a 10% (w/w) substituted hydroxyalkoxypropyl derivative of Sephadex G-25. The assay is commonly used in fatty acid binding protein studies since the derivative non-covalently binds to fatty acids and protein-bound fatty acids. The unique feature of the assay is that unbound and protein-bound fatty acids can be selectively removed from the Lipidex in a temperature dependent manner (17). At 37°C both protein-bound and unbound fatty acids attach to Lipidex. At 0°C only unbound fatty acids remain attached to Lipidex while protein-bound fatty acids are released into solution. The addition of 100 μM Triton X-100 eliminates any nonspecific binding of fatty acids to the surface of the reaction tube (43).

When low concentrations of *C. jejuni* membrane preparations were incubated with PC and Lipidex 1000, a 14 kDa protein was the most abundant protein detected on silver stained gels (Fig. 5.2). Initial attempts to purify the protein by this method were unsuccessful probably due to difficulties in solubilizing the membranes and to nonspecific binding of other components of the preparation to Lipidex.

Serum reactivity against *C. jejuni* whole cell lysates. All serum samples from the LCDC along with controls were tested to determine if they reacted with the 14 kDa

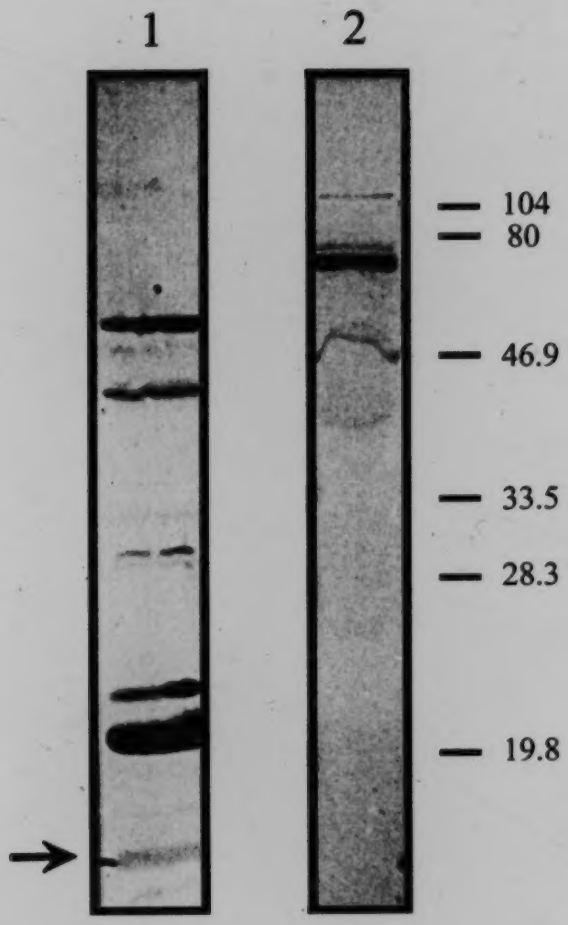


Figure 5.1. Comparison of antibody crossreactivity with *C. jejuni* whole cell lysates. The reaction between *C. jejuni* lysates and anti-myelin P2 antibodies is shown in lane 1. The reaction between *C. jejuni* lysates and anti-nsL-TP antibodies is shown in lane 2. The molecular weights are shown on the right in kDa.

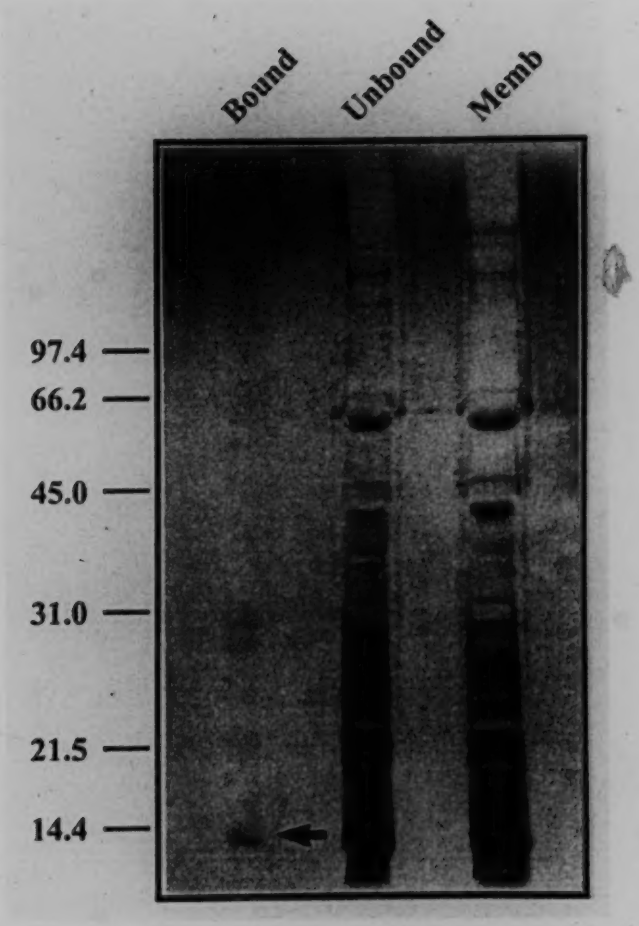


Figure 5.2. Silver stained polyacrylamide gel demonstrating the binding of a 14 kDa *C. jejuni* membrane protein to phosphatidylcholine. The Lipidex 1000 procedure was performed as described in the Materials and Methods section. The *C. jejuni* membrane preparation used in the procedure is shown in (Memb). Proteins which did not bind to the Lipidex are shown in (Unbound). The protein which was eluted from the lipid is shown in (Bound).

protein of *C. jejuni* whole cell lysates (Figure 5.3). Interestingly, all *Campylobacter* positive, GBS positive sera reacted with the 14 kD protein. Most, but not all of the *Campylobacter* positive, GBS negative sera reacted with the protein (sample 42 was negative and sample 53 may also be negative). Neither the GBS positive or Miller Fisher syndrome positive, *Campylobacter* negative sera reacted with the protein. The control lane, which did not contain any human sera (Sec), and the serum sample from a volunteer without previous *Campylobacter* infection (Neg) were also negative. Although only the 14 kDa protein was compared, the blots demonstrated that other *Campylobacter* proteins also exhibited varying reactivities with the sera analyzed.

Serum reactivity against ALBP. All sera were examined to determine whether they reacted with a member of the fatty acid binding protein family, ALBP, kindly donated by Dr. D. A. Bernlohr (Figure 5.4). All samples showed background levels of reactivity to ALBP. This result is not surprising since previous experiments with sera against a proven member of the family, myelin protein P2, also did not react with ALBP. Interestingly, some serum samples reacted with other bands that were not visible in the control. Since ALBP was produced in the recombinant form in *E. coli* it is believed that the reactivity could be against *E. coli* proteins or lipopolysaccharide (Figure 5.4, C39 showed ladder-like patterns). Alternatively, in the case of reactivity to higher molecular weight bands, the serum samples could be reacting with dimers or oligomers of FABP's which are often detected in blots even after the proteins are analyzed on denaturing gels (Bernlohr, personal communication).

However, it may also be possible that the antibodies are recognizing smaller fragments of ALBP. *In vitro* transcription/translation of the RNA of the human liver fatty acid binding protein yielded the 14.3 kDa product along with smaller products (24). The authors of this publication speculated that these smaller products were due to internal translation start codons since the smaller products also reacted with antibodies against the liver protein. In our experiments, the number of lanes showing recognition of smaller

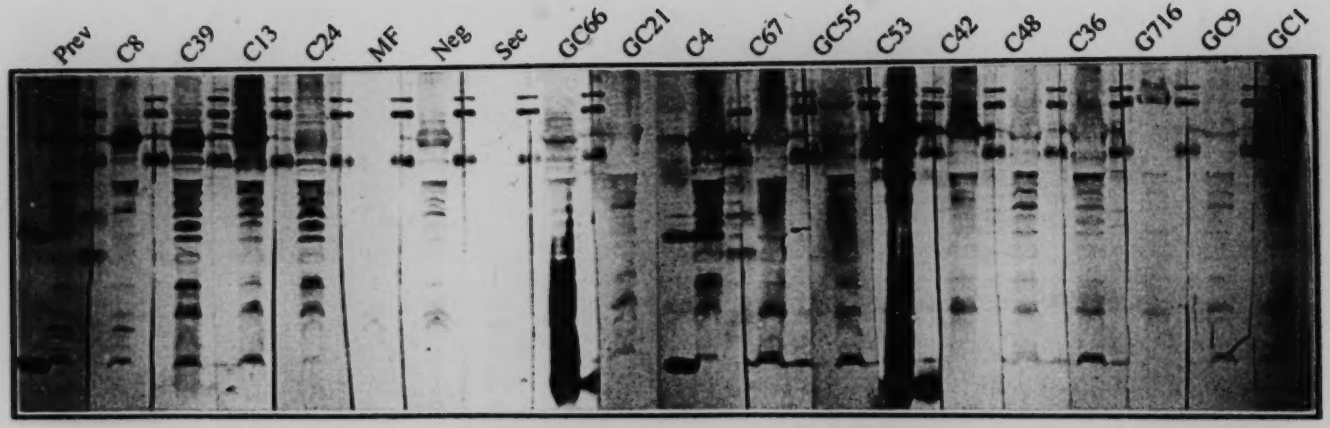


Figure 5.3. Human antibody reactivities to *C. jejuni* whole cell lysates. The lane labelled Prev was incubated with antisera from a volunteer who was previously infected with *C. jejuni*. Neg antisera was obtained from a volunteer that was never infected with *C. jejuni*. MF sera is from a patient with Miller Fisher syndrome without previous knowledge of a *Campylobacter* infection. The lane labelled Sec was not incubated with human antisera thus demonstrating the background reactivity with the secondary antibody. Antisera from patients that tested positive for *C. jejuni* infection are designated by the letter "C". Antisera from patients positive for GBS but negative for *C. jejuni* are designated by the letter "G". Antisera from patients positive for both GBS and *C. jejuni* are designated by the letters "GC". In the first lane of Prev and of C4, the 14.4 and the 31 kDa markers are shown. Prestained high molecular weight markers (Bio-Rad) are shown between each lane.

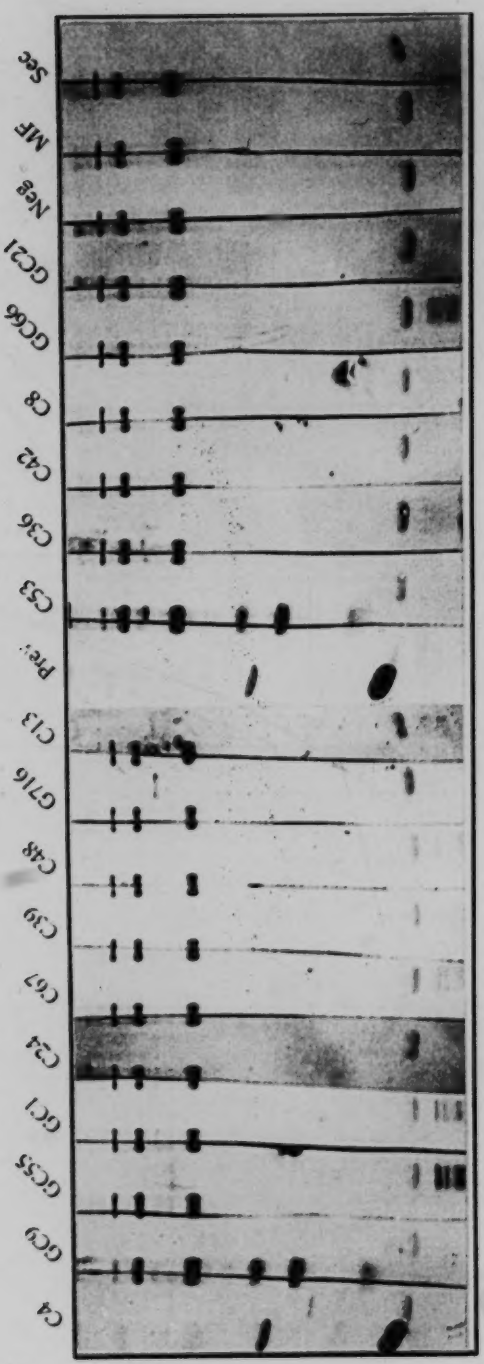


Figure 5.4. Human antibody reactivities to the adipocyte lipid binding protein. Lane descriptions can be found in the figure legend of Figure 5.3.

products, relative to the number of samples in that group, showed the following trend: no sera (0/1), Campylobacter negative (0/1), previous Campylobacter positive (1/1), Campylobacter positive (1/10), Campylobacter negative and GBS / Miller Fisher positive (0/2), Campylobacter positive and GBS positive (3/5).

Thin Layer Chromatography Overlay Analysis of Serum Reactivity to Lipids. The following lipids were spotted on TLC plates: mixed brain gangliosides, GM1 ganglioside, asialo-GM1 ganglioside, GT1 trisialoganglioside, GL4 globoside, neutral glycolipids, cerebrosides, glucocerebrosides, galactocerebrosides, phosphatidylcholine, sphingomyelin, and cardiolipin. No differences were observed in serum reactivities above background levels (Figure 5.5).

5.4. DISCUSSION

There are several well-described protein families which interact with lipids. The proteins involved in lipid transport include: the fatty acid binding proteins (FABP) or lipid binding proteins (LBP) which also include the cellular retinol and retinoic acid binding proteins (CRBP and CRABP, respectively). Another protein family involved in lipid transfer includes the phosphatidylcholine transfer proteins (PC-TP), the phosphatidylinositol transfer proteins (PI-TP) and the non-specific lipid transfer proteins (nsL-TP) also known as sterol carrier protein 2 (SCP2).

Eucaryotic fatty acid binding proteins are generally cytosolic proteins with low molecular masses (14-15 kD) (9). However, several membrane-associated FABP's have been identified and are believed to transport fatty acids across the membrane (11, 12, 41). FABP's are easily identified by their ability to bind to radioactively labelled fatty acids. In addition to binding fatty acids, members of this family have been shown to bind to other hydrophobic compounds. The ileal lipid binding protein (ILBP) is expressed in villus associated enterocytes. ILBP binds to fatty acids with low affinity and demonstrates a preference for bile salts. The liver FABP (L-FABP) binds two molecules of fatty acids and

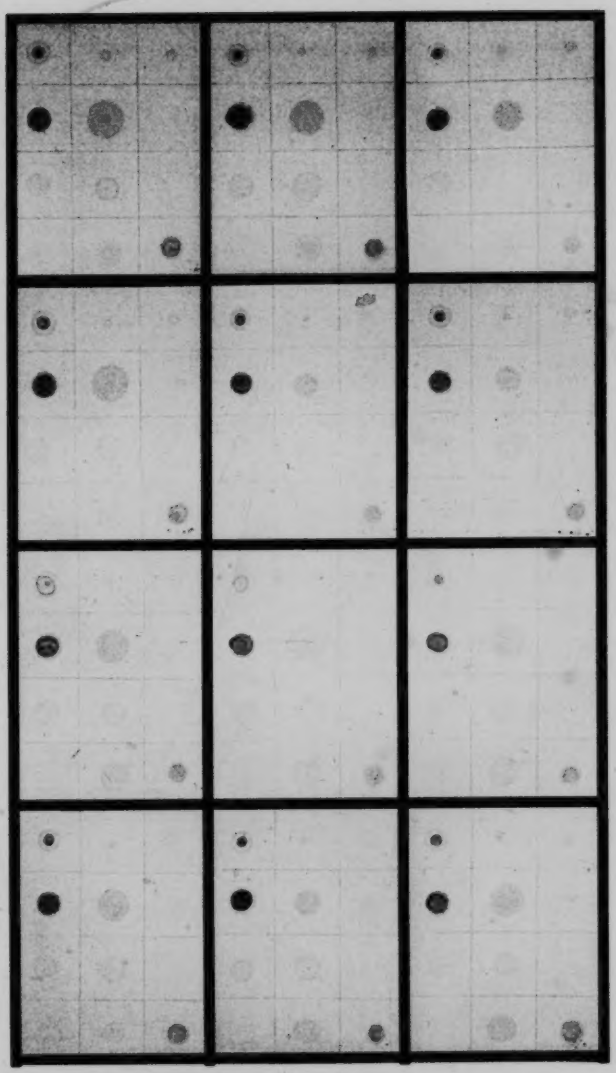


Figure 5.5. TLC overlay comparing antibody responses against various lipids. The lipids spotted from left to right are: mixed brain gangliosides, GM1 ganglioside, asialo-GM1 ganglioside, GT1 trisialoganglioside, GL4 globoside, neutral glycolipids, cerebrosides, glucocerebrosides, galactocerebrosides, phosphatidylcholine, sphingomyelin, and cardiolipin. Sera reacted with the lipids from left to right are: no sera (secondary antibody alone), Neg, Prev, G716, MF, GC66, C67, C42, C53, GC55, GC9, and GC1.

other bulky ligands. L-FABP binds to fatty acids with side-chains, heme, lysophosphatidic acid and some eicosanoids showing preference for ligands with a negatively charged head group. Schroeder *et al.* demonstrated that L-FABP also binds cholesterol (36). The adipocyte lipid binding protein binds to both fatty acids and retinoic acids (25).

E. coli also contains a protein, FadL, which is involved in fatty acid binding. The FadL protein is localized in the outer membrane and may form a specific channel. Although FadL and FABP's have a similar affinity for fatty acids, the authors suggest that it is unlikely that these two proteins exhibit significant homology (7).

Nonspecific lipid transfer proteins, also known as sterol carrier protein 2, stimulate the biosynthesis of cholesterol and accelerate the transfer of both phospholipids and cholesterol between membranes (40). The molecular weights of these proteins also range between 8.8 and 14 kDa (28). In comparison to the FABP's, the nsL-TP's are a distinct class of proteins initially found to bind cholesterol. However there are some structural similarities between the two protein families suggesting an evolutionary conservation (35). FABP's and nsL-TP appear to be ubiquitous proteins present in a variety of species (35).

Antibodies against nsL-TP did not react with the *C. jejuni* 14 kDa protein. However, myelin P2 antisera recognized the 14 kDa protein. We previously showed that *C. jejuni* preferentially binds to unsaturated lipids (see Chapter 3). Also, the 14 kDa *C. jejuni* membrane protein was shown to bind to phosphatidylcholine (PC) using the Lipidex 1000 assay, a procedure commonly used for the FABP's. These results suggest that the *C. jejuni* 14 kDa protein may share functional and antigenic homologies with other FABP's such as myelin P2. Although FABP's have yet to be described in bacteria, it is possible that a similar protein family does exist. Recently, Bishop *et al.* described the first bacterial member of the lipocalin family (6). The lipocalins are a diverse family of proteins which bind and transport small hydrophobic ligands (10). From common sequence motifs and common structural features it is speculated that lipocalins and FABP's share a common

evolutionary relationship (14). Indeed, it has been proposed to put lipocalins and FABP's into a larger superfamily called the calycins (14). Since the two families may share a common evolutionary relationship and since lipocalins have been shown to have originated earlier in evolution than previously imagined, it is quite possible that FABP's may also have as yet unidentified bacterial counterparts.

Myelin P2 is a 14 kDa FABP in myelin and in the cytosol of Schwann cells and oligodendrocytes (5). The myelin P2 protein is found exclusively in myelin sheaths of the peripheral nervous system (PNS) (39). Myelin P2 and myelin basic protein comprise a major portion of the PNS myelin protein (18). Immunization with myelin P2 induces allergic neuritis (15). Since myelin P2 is located on the cytoplasmic side of myelin and Schwann cell membranes, direct antibody-mediated damage is unlikely. However, P2 reactive T-lymphocytes injected into naive animals induce EAN (2). T cells recognize the endogenous proteins (intracellular autoantigens) that are presented on Schwann cells by MHC II (2). Therefore, local antigen presentation and histocompatibility antigen expression is suggested to be involved in the disease.

A similar autoimmune disease known as experimental allergic encephalomyelitis (EAE) is induced by the immunization of animals with myelin basic protein and adjuvant (1). Also, myelin basic protein specific T-cells can induce EAE when injected into naive animals. EAE has been shown to be exclusively mediated by T-cells (1). It is possible that EAN, as well as GBS, is a T-cell mediated autoimmune disorder.

Antiserum from a clinical *C. jejuni* isolate from a GBS patient reacted strongly with myelin protein P0, significantly with myelin P2 and not with myelin P1 (15). These results suggest that some *C. jejuni* proteins share antigenic properties with myelin P0 and P2. Quarles *et al.* also observed antibodies to gangliosides and myelin proteins (P2, P0 and myelin associated glycoprotein-MAG) in GBS patients (29). Our results showed that all sera from GBS patients with previous *C. jejuni* infections reacted strongly with the 14 kDa protein. However, most but not all sera from *C. jejuni* patients without GBS reacted to the

protein. It was also interesting to find that patients with GBS or with Miller Fisher Syndrome without any evidence of a previous *C. jejuni* infection did not react with the 14 kDa protein. These results suggest that the *C. jejuni* 14 kDa protein is immunodominant especially in GBS patients with preceding *Campylobacter* infection. Previous reports have described a 14.5 kDa protein recognized by serum IgG and sIgA present in stools of patients recovering from *C. jejuni* enteritis (45).

However, since it is currently believed that GBS is induced by the development of antibodies to the *C. jejuni* ganglioside-like LPS, we also looked at human serum reactivity to various gangliosides and related structures. It has been reported that gangliosides offer partial protection in EAN (21). In addition to the myelin P2 protein, EAN can be induced by galactocerebroside (19). Also, there is one report of anticardiolipin antibodies in a GBS patient (19). As a result of these reports, we also examined several lipids and glycolipids, in addition to the ganglioside structures suggested to be involved in GBS. We did not observe any differences in serum reactivity to these structures above background levels (Figure 5.5.).

Although there are several reports describing serum antibodies to gangliosides in GBS patients (19), many groups agree that the pathogenic relevance of the antibodies has not yet been established (13, 29, 31, 44). Quarles *et al.* reported that anti-GM1 antibodies were not detected in any of the patients with GBS (29). It has also been shown that human serum normally contains antibodies against gangliosides and other carbohydrate determinants that do not cause tissue damage (37). Patients with *C. jejuni* infection without neurologic disease also had ganglioside antibodies (42). Results from these laboratories suggest that antiganglioside antibodies do not have a significant role in GBS.

Both *C. jejuni* type-strains for serotypes 0:4 and 0:19 contain low molecular weight LPS with terminal sequences mimicking GM1 (tetrasaccharide) and GD1a (pentasaccharide) differing only in the relative proportions of these structures (33, 34). However, there are a lack of reports of GBS cases associated with the frequently isolated

C. jejuni O:4 which also contain these structures. The authors do not believe that a difference in proportions of GM1 and GD1a is related to triggering GBS (4). Also, GBS is found to develop after *C. jejuni* infection with serotype O:2 which does not have either GM1- or GD1a-like structures.

Interestingly, the LPS from two *C. jejuni* isolates from GBS patients (both serotype O:19) differed slightly from the LPS of the serotype O:19 type-strain (3). The first isolate contained an additional sialic acid identical to GT1a (hexasaccharide) while the other strain contained only the terminal trisaccharide identical to GD3. The terminal trisaccharide structure was determined to be common between the GT1a and GD3 cores of the two O:19 isolates from GBS patients as well as in a reported O:2 isolate from a Miller Fisher patient (34). This terminal trisaccharide structure which consisted of two molecules of sialic acid linked to galactose, has not yet been found in non-neuropathogenic isolates. Therefore, the authors suggest that the link between GBS, Miller Fisher syndrome and *C. jejuni* may be the presence of a bacterial α-2,8 sialyltransferase required for the addition of the second sialic acid (34). However, these results still suggest that antibodies to specific portions of gangliosides are involved in precipitating GBS.

A new perspective to the story was provided in a report recently published by Ritter *et al.* (33). The group reported that GM2-ganglioside antibodies are an effective treatment for certain cancer patients and proposes to immunize these patients with LPS from *C. jejuni* GM2-like serotypes. Interestingly, the group also reported that animals immunized with the GM2-like serotypes (O:1, O23, and O:36) *as well as* serotype O:19 did not produce any clinical symptoms. Therefore a role, if any, of ganglioside antibodies in pathogenesis still remains to be determined.

From the experiments described herein, the following relationships were observed: 1) All GBS patients form antibodies against the *C. jejuni* 14 kDa protein. 2) Most *Campylobacter* positive patients (8/10) form antibodies against the 14 kDa protein. 3) Serum samples do not contain antibodies that cross-react with ALBP but do contain

antibodies against unidentified components in the ALBP preparation. 4) Serum samples do not react with any of the lipids tested suggesting lipids, in particular gangliosides, do not have a significant role in GBS. 5) The *C. jejuni* 14 kDa protein is immunodominant. 6) Myelin P2 protein may share some homology with the *C. jejuni* 14 kDa protein.

5.4. REFERENCES

1. **Abbas, A. K., A. H. Lichtman, and J. S. Pober**. 1991. Cellular and molecular immunology. p. 366-367. *In* W. B. Saunders Company, Philadelphia .

2. **Aberer, E., C. Brunner, G. Suchanek, H. Klade, A. Barbour, G. Stanek, and H. Lassmann**. 1989. Molecular mimicry and Lyme Borreliosis: a shared antigenic determinant between *Borrelia burgdorferi* and human tissue. Ann. Neurol. **26:** 732-737.

3. **Aspinall, G. O., S. Fujimoto, M. A. G., H. Pang, L. A. Kurjanczyk, and J. L. Penner**. 1994. Lipopolysaccharides from *Campylobacter jejuni* associated with Guillain-Barré syndrome patients mimic human gangliosides in structure. Infect. Immun. **62:** 2122-2125.

4. **Aspinall, G. O., A. G. McDonald, H. Pang, L. A. Kurjanczyk, and J. L. Penner**. 1994. Lipopolysaccharides of *Campylobacter jejuni* serotype O:19: structures of core oligosaccharide regions from the serostrain and two bacterial isolates from patients with the Guillain-Barré syndrome. Biochem. **33:** 241-249.

5. **Baron, P., G. Constantin, A. D'Andrea, D. Ponzin, E. Scarpini, G. Scarlato, G. Trinchieri, F. Rossi, and M. A. Cassatella**. 1993. Production of tumor necrosis factor and other proinflammatory cytokines by human mononuclear phagocytes stimulated with myelin P2 protein. Proc. Natl. Acad. Sci. USA. **90:** 4414-4418.

6. **Bishop, R. E., S. S. Penfold, L. S. Frost, J.-V. Höltje, and J. H. Weiner**. 1995. Stationary phase expression of a novel *Escherichia coli* outer membrane lipoprotein and its relationship with mammalian apolipoprotein D - implications for the origin of lipocalins. J. Biol. Chem. **270:** 23097-23103.

7. **Black, P. N.** 1990. Characterization of FadL-specific fatty acid binding in *Escherichia coli*. Biochim. Biophys. Acta. **1046:** 97-105.

8. **Bolton, C. F.** 1995. The changing concepts of Guillain-Barré syndrome. N Engl J Med. **333:** 1415-1417.

9. **Börchers, T., and F. Spener**. 1993. Involvement of arginine in the binding of heme and fatty acids to fatty acid-binding protein from bovine liver. Molec. Cell. Biochem. **123:** 23-27.

10. **Börchers, T., and F. Spener**. 1994. Fatty acid binding proteins, p. 261-294. *In* Hoekstra, D. (ed.), Cell Lipids. Academic Press Inc., San Diego .

11. **Campbell, F. M., and A. K. Dutta-Roy**. 1995. Plasma membrane fatty acid-binding protein ($FABP_{pm}$) is exclusively located in the maternal facing membranes of the human placenta. FEBS Lett. **375:** 227-230.

12. **Campbell, F. M., M. J. Gordon, and A. K. Dutta-Roy**. 1994. Plasma membrane fatty acid-binding protein ($FABP_{pm}$) of the sheep placenta. Biochim. Biophys. Acta. **1214:** 187-192.

13. **Enders, U., H. Karch, K. V. Toyka, M. Michels, J. Zielasek, M. Pette, J. Heesemann, and H.-P. Hartung**. 1993. The spectrum of immune responses to *Campylobacter jejuni* and glycoconjugates in Guillain-Barré syndrome and in other neuroimmunological disorders. Ann. Neurol. **34:** 136-144.

14. **Flower, D. R., A. C. T. North, and T. K. Attwood**. 1993. Structure and sequence relationships in the lipocalins and related proteins. Protein Sci. **2:** 753-761.

15. **Fujimoto, S., and K. Amako**. 1990. Guillain-Barré syndrome and *Campylobacter jejuni* infection. Lancet. **335:** 1350.

16. **Fujimoto, S., N. Yuki, T. Itoh, and K. Amako**. 1992. Specific serotype of *Campylobacter jejuni* associated with Guillain-Barré syndrome. J. Infect. Dis. **165:** 183.

17. **Glatz, J. F. C., and J. H. Veerkamp**. 1983. A radiochemical procedure for the assay of fatty acid binding by proteins. Anal. Biochem. **132:** 89-95.

18. **Hayasaka, K., K. Nanao, M. Tahara, W. Sato, G. Takada, M. Miura, and K. Uyemura**. 1991. Isolation and sequence determination of cDNA encoding P2 protein of human peripheral myelin. Biochem. Biophys. Res. Commun. **181:** 204-207.

19. **Ilyas, A. A., H. J. Willison, R. H. Quarles, F. B. Jungalwala, D. R. Cornblath, B. D. Trapp, D. E. Griffin, J. W. Griffin, and G. M. McKhann**. 1988. Serum antibodies to gangliosides in Guillain-Barré syndrome. Ann. Neurol. **23:** 440-447.

20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227:** 680-685.

21. **Ledeen, R. W., B. Oderfeld-Nowak, C. F. Brosnan, and A. Cervone.** 1990. Gangliosides offer partial protection in experimental allergic neuritis. Ann. Neurol. **27:** S69-S74.

22. **Lingwood, C. A., M. Huesca, and A. Kuksis.** 1992. The glycerolipid receptor for *Helicobacter pylori* (and exoenzyme S) is phosphatidylethanolamine. Infect. Immun. **60:** 2470-2474.

23. **Lingwood, C. A., H. Law, A. Pellizzari, P. Sherman, and B. Drumm.** 1989. Gastric glycerolipid as a receptor for *Campylobacter pylori*. Lancet. **2:** 238-241.

24. **Maatman, R. G. H. J., E. M. A. van de Westerlo, T. van Kuppevelt, H. M. S. M., and J. H. Veerkamp.** 1992. Molecular identification of the liver- and the heart-type fatty acid-binding proteins in human and rat kidney: use of the reverse transcriptase polymerase chain reaction. Biochem. J. **288:** 285-290.

25. **Matarese, V., and D. A. Bernlohr.** 1988. Purification of murine adipocyte lipid-binding protein- characterization as a fatty acid- and retinoic acid-binding protein. J. Biol. Chem. **263:** 14544-14551.

26. **Mishu, B., and M. J. Blaser.** 1993. Role of infection due to *Campylobacter jejuni* in the initiation of Guillain-Barré syndrome. Clin. Infect. Dis. **17:** 104-108.

27. **Mishu-Allos, B., and M. J. Blaser**. 1995. *Campylobacter jejuni* and the expanding spectrum of related infections. Clin. Infect. Dis. **20:** 1092-1099.

28. **Ossendorp, B. C., G. T. Snoek, and K. W. A. Wirtz**. 1994. Intracellular phospholipid transfer proteins, p. 217-259. *In* Hoekstra, D. (ed.), Cell Lipids. Academic Press Inc., San Diego .

29. **Quarles, R. H., A. A. Ilyas, and H. J. Willison**. 1990. Antibodies to gangliosides and myelin proteins in Guillain-Barré syndrome. Ann. Neurol. **27:** S48-S52.

30. **Rees, J. H., N. A. Gregson, P. L. Griffiths, and R. A. C. Hughes**. 1993. *Campylobacter jejuni* and Guillain-Barré syndrome. Quart. J. Med. **86:** 623-634.

31. **Rees, J. H., N. A. Gregson, and A. C. Hughes**. 1995. Anti-ganglioside GM1 antibodies in Guillain-Barré syndrome and their relationship to *Campylobacter jejuni* infection. Annal. Neurol. **38:** 809-816.

32. **Rees, J. H., S. E. Soudain, N. A. Gregson, and R. A. C. Hughes**. 1995. *Campylobacter jejuni* infection and Guillain-Barré syndrome. New Engl. J. Med. **333:** 1374-1379.

33. **Ritter, G., S. R. Fortunato, L. Cohen, L. Noguchi, E. M. Bernard, E. Stockert, and L. J. Old**. 1996. Induction of antibodies reactive with GM2 ganglioside after immunization with lipopolysaccharides from *Campylobacter jejuni*. Int. J. Cancer. **66:** 184-190.

34. **Salloway, S., L. A. Mermel, M. Seamans, G. O. Aspinall, J. E. Nam Shin, L. A. Kurjanczyk, and J. L. Penner**. 1996. Miller-Fisher syndrome associated with *Campylobacter jejuni* bearing lipopolysaccharide molecules that mimic human ganglioside GD3. Infect. Immun. **64:** 2945-2949.

35. **Sams, G. H., B. M. Hargis, and P. S. Hargis**. 1991. Identification of two lipid binding proteins from liver of *Gallus domesticus*. Comp. Biochem. Physiol. **99B:** 213-219.

36. **Schroeder, F., J. R. Jefferson, D. Powell, S. Incerpi, J. K. Woodford, S. M. Colles, S. Myers-Payne, T. Emge, T. Hubbell, D. Moncecchi, D. R. Prows, and C. E. Heyliger**. 1993. Expression of rat L-FABP in mouse fibroblasts: role in fat absorption. Molec. Cell. Biochem. **123:** 73-83.

37. **Svennerholm, L., and P. Fredman**. 1990. Antibody detection in Guillain-Barré syndrome. Ann. Neurol. **27:** S36-S40.

38. **Szymanski, C. M., and G. D. Armstrong**. 1996. Interactions between *Campylobacter jejuni* and lipids. Infect. Immun. **64:** 3467-3474.

39. **Trapp, B. D., L. J. McIntyre, R. H. Quarles, N. H. Sternberger, and H. D. Webster**. 1979. immunocytochemical localization of rat peripheral nervous system myelin proteins: P_2 protein is not a component of all peripheral nervous system myelin sheaths. Proc. Natl. Acad. Sci. USA. **76:** 3552-3556.

40. **van Amerongen, A., M. van Noort, J. R. C. M. van Beckhoven, F. F. G. Rommerts, J. Orly, and K. W. A. Wirtz**. 1989. The subcellular distribution of

the nonspecific lipid transfer protein (sterol carrier protein 2) in rat liver and adrenal gland. Biochim. Biophys. Acta. **1001:** 243-248.

41. **Van Nieuwenhoven, F. A., G. J. Van der Vusse, and J. F. C. Glatz**. 1996. Membrane-associated and cytoplasmic fatty acid-binding proteins. Lipids. **31:** S-223-S-227.

42. **von Wulffen, H., C. Hartard, and E. Scharein**. 1994. Seroreactivity to *Campylobacter jejuni* and gangliosides in patients with Guillain-Barré syndrome. J. Infect. Dis. **170:** 828-833.

43. **Vork, M. M., J. F. C. Glatz, D. A. M. Surtel, and G. J. van der Vusse**. 1990. Assay of the binding of fatty acids by proteins: evaluation of the Lipidex 1000 procedure. Mol. Cell. Biochem. **98:** 111-117.

44. **Vriesendorp, F. J., B. Mishu, M. J. Blaser, and C. L. Koski**. 1993. Serum antibodies to GM1, GD1b, peripheral nerve myelin, and *Campylobacter jejuni* in patients with Guillain-Barré syndrome and controls: correlation and prognosis. Ann. Neurol. **34:** 130-135.

45. **Wu, Y. L., L. H. Lee, D. M. Rollins, and W. M. Ching**. 1994. Heat shock- and alkaline pH-induced proteins of *Campylobacter jejuni*: characterization and immunological properties. Infect. Immun. **62:** 4256-4260.

46. **Yuki, N., S. Handa, T. Tai, M. Takahashi, K. Saito, Y. Tsujino, and T. Taki**. 1995. Ganglioside-like epitopes of lipopolysaccharides from *Campylobacter*

jejuni (PEN 19) in three isolates from patients with Guillain-Barré syndrome. J. Neurol. Sci. **130:** 112-116.

CHAPTER 6

CAMPYLOBACTER JEJUNI BINDING AND INVASION

6.1. INTRODUCTION

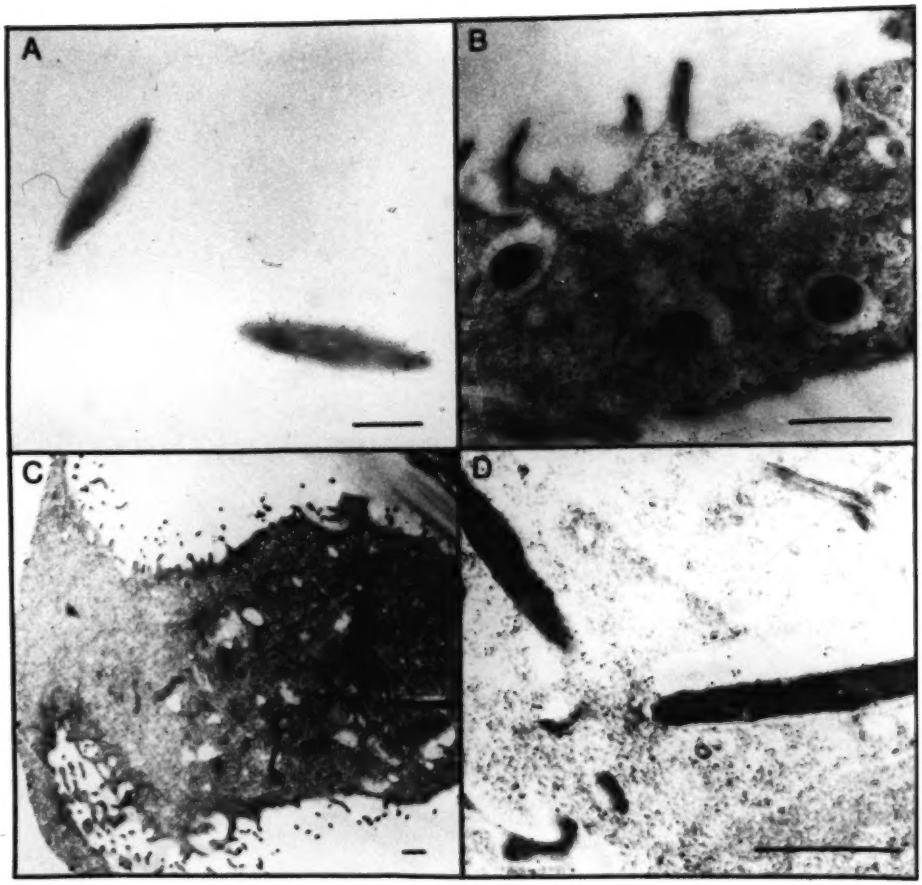
This chapter will be divided into two sections: control experiments and signal transduction experiments. The experiments described in the former section were necessary to set the foundation for several of the studies described in the previous chapters. The latter section deals with investigations of signal transduction mechanisms involved in *C. jejuni* infection.

6.2. RESULTS AND DISCUSSION

6.2.1. CONTROL EXPERIMENTS

Immunoelectron microscopy was used to determine whether antibodies raised against the porin protein of *C. jejuni* UA580 reacted with the organism. *C. jejuni* UA580 was adsorbed onto Formvar coated copper grids. After treating these grids with BSA to block non-specific protein binding they were incubated with antibodies against the *C. jejuni* major outer membrane protein (MOMP). The grids were then washed and incubated with anti-rabbit IgG coupled to gold particles. After removing the unbound gold conjugate, the grids were allowed to air dry and were observed in the transmission electron microscope. *C. jejuni* MOMP-antibody was determined to react with *C. jejuni* UA580 (Figure 6.1.A). Therefore *C. jejuni* UA580 infected Caco-2 cells were prepared for immunogold labelling to determine if the strain is invasive. Infected Caco-2 cells were prepared for immunogold labelling by first fixing the cells in 2% glutaraldehyde (v/v) and 2% formaldehyde (v/v) in 100 mM sodium phosphate buffer, pH 7.3. The cells were then washed in phosphate buffered saline and gradually dehydrated with ethanol. The samples were cured in K4M solution prepared as described in the kit protocol (1.35 g crosslinker A, 8.65 g monomer

Figure 6.1. Immunogold labelling of *C. jejuni* UA580 with anti-major outer membrane protein antibodies. (A) Positive labelling of *C. jejuni* UA580. (B-D) *C. jejuni* UA580 within Caco-2 cells. Panel D is an enlargement of panel C. The solid bar in each panel represents 1 μM.


A
B
C
D

B, and 0.05 g benzoin ethylether) for several days under ultraviolet light and CO_2. The samples were then sectioned and incubated with anti-*C. jejuni* MOMP antibodies coupled to gold particles. Photographs of transmission electron micrographs demonstrating the internalization of *C. jejuni* UA580 in Caco-2 cells are shown in Figure 6.1.B,C,D.

Since 1% Triton X-100 (v/v) was used in the invasion assays to release intracellular bacteria from infected cells, it was necessary to determine the effect of detergent on *C. jejuni* viability. Bacteria were incubated with 1%, 0.1%, and 0% Triton X-100 (v/v) for 5 min. in a CO_2 incubator as would occur during a gentamicin invasion assay. Then, 800 µl of brain heart infusion broth was added to the wells and serial dilutions were spread onto brain heart infusion agar plates. The viability of *C. jejuni* in the different concentrations of Triton X-100 did not vary significantly (control vs. 0.1%: $p=0.598$; control vs 1.0%: $p=0.667$) (Figure 6.2). The experiment was done once. It was also verified that *C. jejuni* UA580 was gentamicin sensitive by the disc inhibition assay.

C. jejuni UA580 invasion in Caco-2 cells was evaluated at 1, 2, 3, 4, and 5 hours post infection (Figure 6.3). The experiment was done once. It was determined that invasion increased over time with the highest levels of invasion at 5 hours post infection. However, since invasion rates beyond 5 hours were not determined, it is unknown whether the rates continue to increase or reach a maximum beyond 5 hours post infection. In INT 407 cells, Konkel *et al.* reported that the greatest number of invasive organisms was found at 6 hours post infection for all three *C. jejuni* isolates investigated (5).

6.2.2. SIGNAL TRANSDUCTION EXPERIMENTS

Several pathogenic microorganisms usurp the host cell cytoskeleton for their own use. Cytoskeletal manipulation may be in the form of bacterial binding pedestals known as attaching and effacing lesions formed by organisms such as enteropathogenic *E. coli* (EPEC) and *Helicobacter pylori*. While organisms such as *Salmonella* and *Yersinia* utilize the host cytoskeleton to invade host cells. Also, *Salmonella typhimurium* has recently

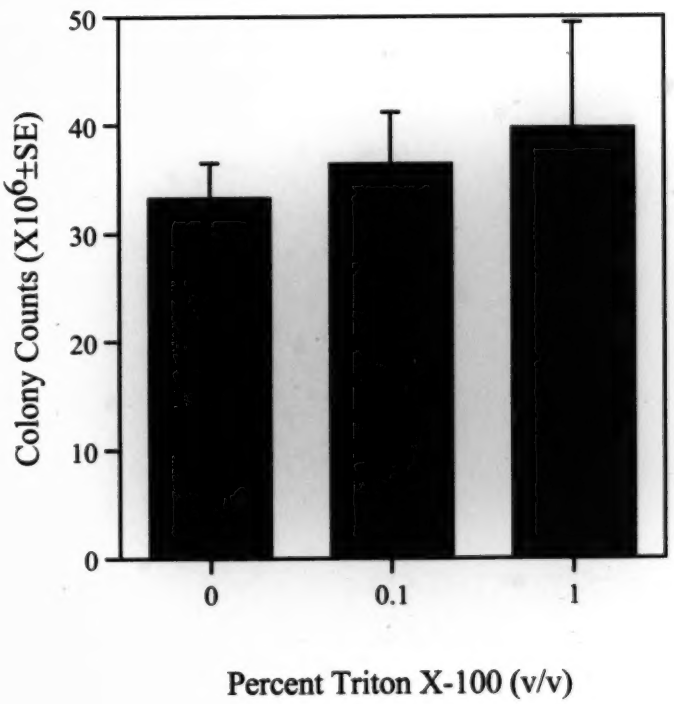


Figure 6.2. Effect of triton X-100 on the viability of *C. jejuni* E863. The data represent results from one experiment. T-tests showed that triton X-100 did not significantly alter *C. jejuni* viability (0.1%: p=0.598; 1.0%: p=0.667).

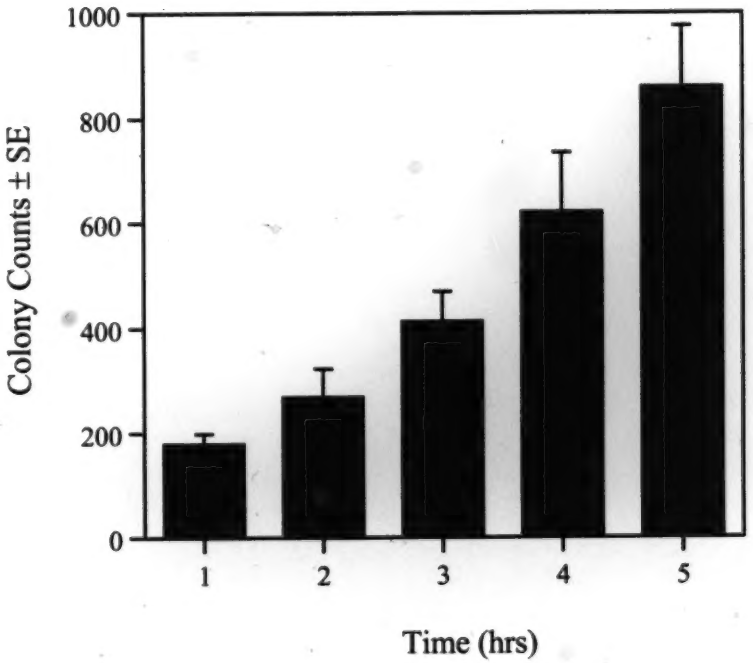


Figure 6.3. *C. jejuni* UA580 invasion timecourse in Caco-2 cells. The data shown in this graph were determined from one experiment.

been shown to induce actin rearrangements which lead to the formation of host cell membrane ruffles (3). Other organisms such as *Shigella* and *Listeria* have been shown to use the host cytoskeleton for invasion, intracellular movement and cell-to-cell spread. Microfilaments and / or microtubules have been shown to play a role in *C. jejuni* invasion (5-7). We examined the roles of two cytoskeletal inhibitors: cytochalasin D and colchicine. Cytochalasin D prevents actin from polymerizing into microfilaments while colchicine prevents tubulin from polymerizing into microtubules. The binding and invasion assays were done as described in Chapter 2. However, cytochalasin D and colchicine were incubated with the cell monolayers for 1 hr prior to infection and the incubation and infection were done in the dark due to the light sensitivity of the compounds. Caco-2 cells were infected with *C. jejuni* E863 for 5 hrs in the presence of no inhibitor (control), or in the presence of 5, 10, or 20 μM colchicine or cytochalasin D (Figure 6.4). Colchicine and cytochalasin D inhibited *C. jejuni* invasion in a concentration dependent manner. This result suggested that invasion was dependent on host cell microfilaments as well as microtubules. However, the inhibitors did not significantly change *C. jejuni* binding to Caco-2 cells (Figure 6.5). Konkel *et al.* observed slight increases in *C. jejuni* binding to HEp-2 cells in the presence of cytochalasin B (6).

Table 6.1 summarizes the reported effects of microfilament inhibitors (cytochalasin B and cytochalasin D) and microtubule inhibitors (colchicine, vincristine, and vinblastine) on *C. jejuni* infection. It is evident from the table that experiments with cytoskeletal inhibitors do not give consistent results. Russell and Blake suggested that the differences observed may be due to differences in cell lines or strains used (8). It is evident from the work of Oelschlaeger *et al.* that strain differences do exist since invasion of only one of the two *C. jejuni* strains tested was inhibited by cytochalasin D (7). Russell and Blake also suggested that at high inhibitor concentrations, other cellular mechanisms may be inhibited. Also, Tang *et al.* mentioned that colchicine may have other effects (9) while they suggested that cytochalasin D is the most potent and specific of the cytochalasins (9). We also

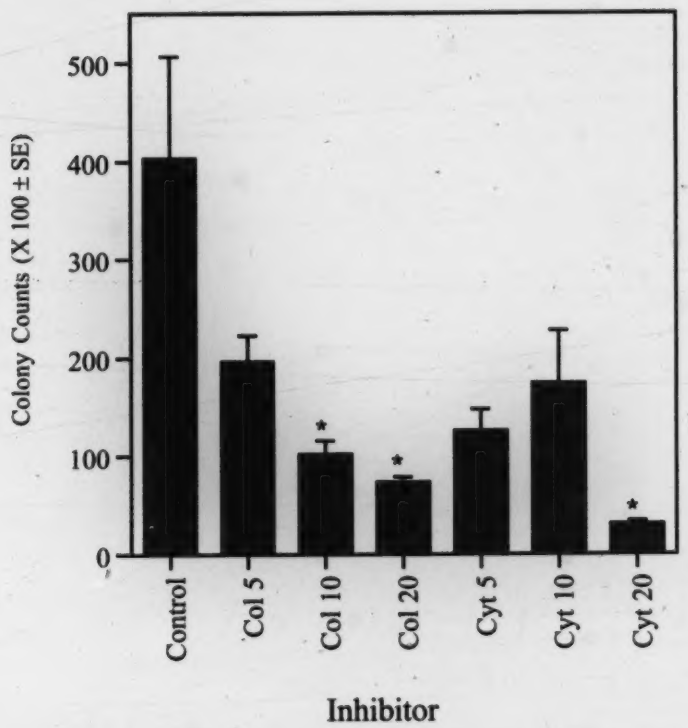


Figure 6.4. *C. jejuni* E863 invasion of Caco-2 cells in the presence of cytoskeletal inhibitors. Invasion assays were done in the presence of the inhibitors colchicine (Col) and cytochalasin D (Cyt) at the indicated concentrations (μM). The control and Col 10 values were determined from quadruplicate experiments while the value for Cyt 10 was determined from triplicate experiments. The remaining data were determined from single experiments. The asterisks represent invasion values that are significantly different from the control by the t-test (Col 5: p=0.507; Col 10: p=0.02; Col 20: p=0.031; Cyt 5: p=0.121; Cyt 10: p= 0.160; Cyt 20: p=0.019).

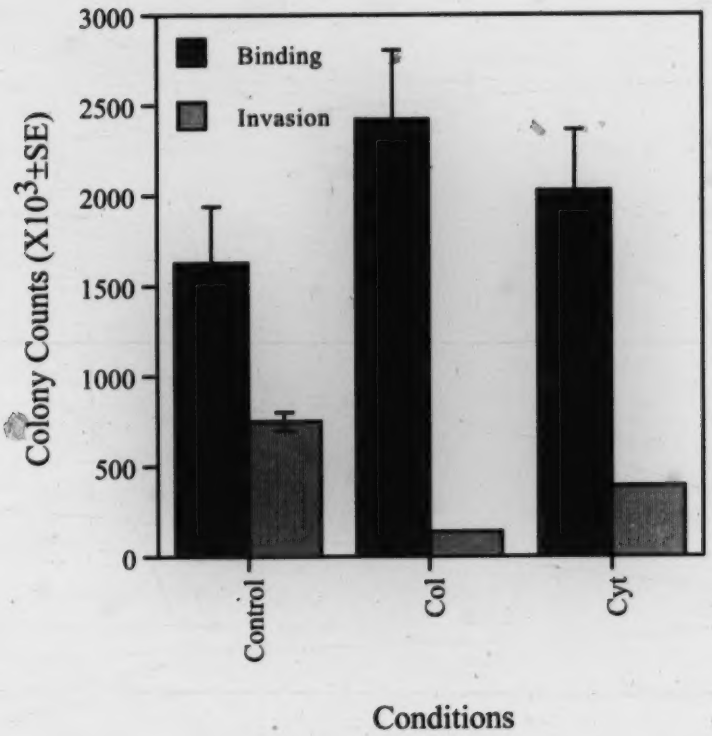


Figure 6.5. *C. jejuni* E863 binding and invasion of Caco-2 cells in the presence of cytoskeletal inhibitors. The final concentration of both colchicine (Col) and cytochalasin D (Cyt) was 10 μM. The binding and invasion data were determined from duplicate experiments. T-test comparisons gave the following results: Control binding vs. Col: p=0.638; Control binding vs. Cyt: p=0.149; Control invasion vs. Col: p= 0.038; Control invasion vs. Cyt: p=0.084.

TABLE 6.1. COMPARISON OF CYTOSKELETAL INHIBITOR EXPERIMENTS.

Compound	Concentration	Cell Line	Effect	Assay Conditions	Reference
Cytochalasin B	0.5-8.0 μg/ml	HEp-2	binding increased and invasion decreased	added with bacteria and present during 3hr infection	(6)
Cytochalasin D	2 μM and 10 μM	INT 407	invasion decreased (Cyt)	added 45 min. prior and	(5)
Colchicine	2.5, 6.25, 12.5 μM		invasion variable (Col)	present during 3hr infection	
Cytochalasin B	1 μg/ml	Caco-2	no effects on binding	added 1 and? 10 μg/ml of both	(8)
Cytochalasin D	2 μM		or invasion	cytochalasins 30 min. prior	
Vincristine	5 μM			added with bacteria?	
Vinblastine	5 μM			all present during 3hr infection	
Cytochalasin D	2 μM	INT 407	invasion decreased	added 1 hr prior and	(7)
Colchicine	10 μM			present during 2hr infection	
Cytochalasin D	5, 10, 20 μM	Caco-2	no effects on binding	added 1 hr prior and	Chap. 6
Colchicine	5, 10, 20 μM		while invasion decreased	present during 5hr infection	

suggest that differences may be due to the incubation conditions used as well as to the activity differences of inhibitors between shipments. The results reported in this section represent initial experiments done with cytochalasin D. Our later experiments with a new vial of cytochalasin D required higher concentrations of the inhibitor to observe the same effects. The incubation conditions reported by Russell and Blake are unclear (8). This is unfortunate since they are the only group which demonstrated that cytoskeletal inhibitors had no effect on *C. jejuni* invasion in Caco-2 cells.

Once bound, *C. jejuni* appears to be internalized by a microtubule and/or microfilament dependent mechanism. This is unlike other invasive enteric pathogens such as *Salmonella* spp.and *Shigella* spp. which utilize only microfilaments (2). Infection may trigger several host cell responses which may lead to the induction of inflammatory colitis or secretory diarrhea. These mechanisms have yet to be determined. Kaur *et al.* demonstrated that calcium and protein kinase C (PKC), *in vivo*, are important in *C. jejuni* induction of intestinal fluid accumulation (4). Phorbol-12-myristate-13-acetate (PMA) alone induced fluid accumulation in ligated rat ileal loops similar to that induced by *C. jejuni* alone (4). However, the authors did not see a difference in fluid accumulation when PMA was injected with *C. jejuni*. From their results, Kaur *et al.* concluded that PKC is activated during *C. jejuni* infection.

PMA activates PKC by substituting for diacylglycerol (DAG) thereby increasing the affinity for calcium. In one experiment with 20 ng/ml PMA we observed significant inhibition of *C. jejuni* invasion ($p=0.046$). However, since this experiment was not repeated, our *in vitro* results with PMA are inconclusive.

Dantrolene or 1-{[5-(p-nitrophenyl)furfurylidene]amino}hydantoin sodium hydrate inhibits PKC by suppressing, but not inhibiting, calcium release from inositol triphosphate sensitive stores in the endoplasmic reticulum. The compound is also reported to inhibit actin accumulation. Lipid A is another DAG analogue which acts as one of the two compounds necessary to activate PKC (the other is phospholipids) resulting in protein

phosphorylation and host cell response. We determined the effect of dantrolene (in DMSO) and lipid A on the invasion of *C. jejuni* in Caco-2 cells. Dantrolene (50 μM) inhibited invasion (p=0.058) whereas DMSO alone had no effect. Lipid A showed no effect (p=0.399) on invasion. Another potent specific inhibitor of PKC, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7, 15 μg/ml) did not inhibit *C. jejuni* invasion of Caco-2 cells (p=0.291). Kaur *et al.* reported that 15 μg/ml concentrations of H-7 reduced *C. jejuni* induced fluid accumulation in ligated rat ileal loops (4). Our results simply demonstrated that the compound had no effect on *C. jejuni* invasion *in vitro*.

EGTA, a specific chelator of external calcium, was also investigated. Interestingly, 4mM EGTA increased *C. jejuni* invasion by 15-fold. The effect of EGTA may warrant further investigation. Baldwin *et al.* examined the effects of 50 μM dantrolene and 4 mM EGTA on the intracellular free calcium levels in Hep-2 cells infected with EPEC (1). The authors found that dantrolene inhibited the induction of intracellular free calcium levels by EPEC. However, EGTA, had no effect on intracellular calcium induction. Tang *et al.* suggested using lower working concentrations of EGTA (200 μM). However, their experiments were done in calcium free media unlike our experiments and those of Baldwin *et al.* (9)..

We also demonstrated that the calcium channel blocker verapamil significantly inhibited *C. jejuni* E863 invasion of Caco-2 cells in a concentration dependent manner (Figure 6.6). Strangely, verapamil also significantly inhibited *C. jejuni* E863 binding to Caco-2 cells to the same extent (Figure 6.7). We wondered why a calcium channel blocker would inhibit bacterial binding. Since we had also shown that 100 μM verapamil significantly inhibited *C. jejuni* UA580 invasion of Caco-2 cells (p=0.001) and had antibodies against the *C. jejuni* UA580 MOMP, we decided to determine if the organism itself binds to verapamil in an ELISA assay. Microtiter wells were coated with 12.5, 25,

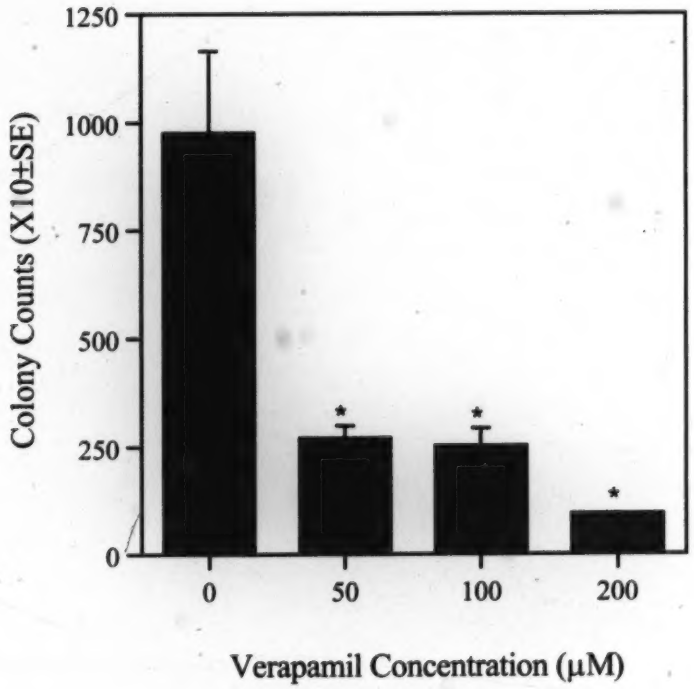


Figure 6.6. *C. jejuni* E863 Caco-2 cell invasion in the presence of verapamil. *C. jejuni* invasion at 0 and 100 μM verapamil was determined from triplicate experiments. *C. jejuni* invasion at 50 and 200 μM verapamil was determined from a single experiment. The asterisks represent significantly different invasion values relative to the control (50 μM: p=0.001; 100 μM: p=0.000; 200 μM: p=0.001).

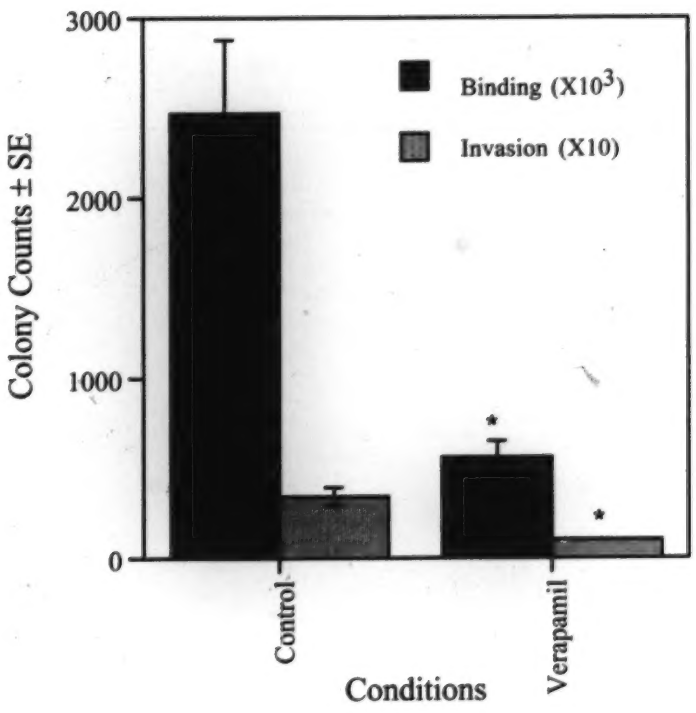


Figure 6.7. *C. jejuni* E863 binding and invasion of Caco-2 cells in the presence of 100 μM verapamil. The data shown were determined from one experiment. The asterisks represent significantly different values from the controls (binding: p=0.014; invasion p=0.003).

50, and 100 μM verapamil and incubated with *C. jejuni* UA580. The experiment, done in triplicate, is shown in Figure 6.8. *C. jejuni* bound to verapamil in a concentration dependent manner. Interestingly, Kaur *et al.* also reported that verapamil (100 μM) reduced *C. jejuni* induced fluid accumulation in ligated rat ileal loops (4). In light of our findings, it is possible that verapamil reduces *C. jejuni* induced fluid accumulation by reducing the amount of organisms that are able to bind to the intestinal epithelium. A reduction in *C. jejuni* binding would lead to a reduction in the amount of actual signal for fluid accumulation.

The toxicity of colchicine, cytochalasin D, PMA, dantrolene, and verapamil was examined. Colchicine, cytochalasin D, and verapamil were not toxic to *C. jejuni* at the concentrations used in the experiments. The toxicity results for PMA are inconclusive. Dantrolene was determined to effect bacterial viability. This is in contrast to the results by Baldwin *et al.* which reported that "bacteria (EPEC) attached and grew normally on dantrolene-treated cells" (1). A summary of the results for the signal transduction experiments is found in Table 6.2.

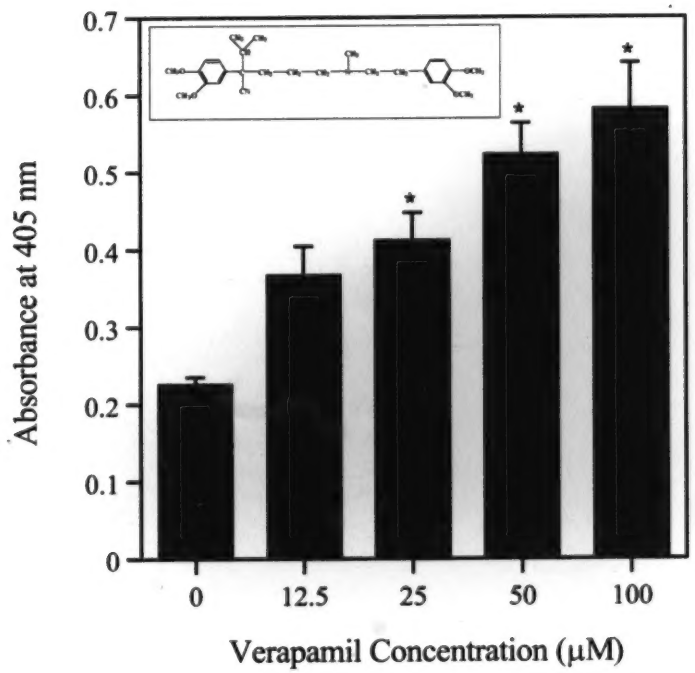


Figure 6.8. *C. jejuni* UA580 binding to verapamil-coated wells. *C. jejuni* binding to 0, 25, and 50 μM verapamil concentrations was determined from triplicate experiments ± SE. *C. jejuni* binding to 12.5 and 100 μM verapamil was determined from duplicate experiments. The asterisks represent absorbance values significantly different from the blank (12.5 μM: p=0.120; 25 μM: p=0.010; 50 μM: p=0.001; 100 μM: p=0.016). The structure of verapamil is shown in the inset.

TABLE 6.2. SUMMARY OF REAGENTS USED AND OBSERVED EFFECTS ON *C. JEJUNI* BINDING AND INVASION

Compound	Activity	Effect	Toxicity	Comments
Colchicine	depolymerizes microtubules	no change in binding invasion decreases	not toxic	role in invasion
Cytochalasin D	depolymerizes microfilaments	no change in binding invasion decreases	not toxic	role in invasion
Verapamil	calcium channel blocker	binding decreases invasion decreases	not toxic	*C. jejuni* binds to compound
H-7	specific PKC inhibitor	no change	not determined	no role
PMA	PKC activator	inconclusive	inconclusive	role not established
Dantrolene	PKC inhibitor (calcium release)	invasion decreases	toxic to C. jejuni	unknown
Lipid A	PKC activator	no change	not determined	no role
EGTA	external calcium chelator	invasion increases	not determined	unknown

6.3. REFERENCES

1. **Baldwin, T. J., W. Ward, A. Aitken, S. Knutton, and P. H. Williams**. 1991. Elevation of intracellular free calcium levels in HEp-2 cells infected with enteropathogenic *Escherichia coli*. Infect. Immun. **59:** 1599-1604.

2. **Groisman, E. A., and H. Ochman**. 1993. Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri*. EMBO J. **12:** 3779-3787.

3. **Jones, B. D., H. F. Paterson, A. Hall, and S. Falkow**. 1993. *Salmonella typhimurium* induces membrane ruffling by a growth factor-receptor-independent mechanism. Proc. Natl. Acad. Sci. USA. **90:** 10390-19394.

4. **Kaur, R., N. K. Ganguly, L. Kumar, and B. N. S. Walia**. 1993. Studies on the pathophysiological mechanism of *Campylobacter jejuni*-induced fluid secretion in rat ileum. FEMS Microbiol. Lett. **111:** 327-330.

5. **Konkel, M. E., S. F. Hayes, L. A. Joens, and W. Cieplak Jr.** 1992a. Characteristics of the internalization and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures. Microbial Pathog. **13:** 357-370.

6. **Konkel, M. E., and L. A. Joens**. 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect. Immun. **57:** 2984-2990.

7. **Oelschlaeger, T. A., P. Guerry, and D. J. Kopecko**. 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. Proc. Natl. Acad. Sci. **90:** 6884-6888.

8. **Russell, R. G., and J. D. C. Blake**. 1994. Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. Infect. Immun. **62:** 3773-3779.

9. **Tang, P., V. Foubister, M. Graciela Pucciarelli, and B. B. Finlay**. 1993. Methods to study bacterial invasion. J. Microbiol. Meth. **18:** 227-240.

CHAPTER 7

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Although the importance of *C. jejuni* in causing disease has been realized for several years, the *Campylobacter* literature is still in its infancy compared to the literature for other known enteric pathogens such as *Escherichia coli*, *Shigella*, and *Salmonella*. In particular, very little is known about the pathogenic mechanisms of *C. jejuni*. Research has been hindered by the lack of available basic genetic manipulations and the lack of inexpensive animal models that are representative of human disease. The work presented in this thesis made a small contribution to the knowledge of *C. jejuni* pathogenesis. The direction for each chapter will be discussed followed by a general summary.

In chapter 2, we examined factors which effect *C. jejuni* motility, binding and invasion. Although *Campylobacter* motility has been extensively studied, very little is known about the attachment and invasion process. For instance, what does *C. jejuni* bind to on host cells? The receptor for *C. jejuni* has yet to be identified. Also, what is the *C. jejuni* adhesin responsible for attachment? Do the newly identified pili play a role in attachment?

In developed countries, *Campylobacter* enteritis is reminiscent of an invasive disease. Yet, the importance of invasion has not been established. Also, the number of organisms that actually invade host cells is rather low. Is invasion important in *C. jejuni* pathogenesis? What is the role of invasion in the disease process? How does *C. jejuni* signal phagocytosis by host cells?

In chapter 3 we demonstrated that *C. jejuni* binds to lipids and lipid derivatives with different affinities. We later showed that *C. jejuni* possesses different proteins that may be involved in lipid interactions. However, it would be interesting to determine if lipids actually mediate *C. jejuni* attachment to host cells.

In chapter 4 we began to characterize the *C. jejuni* proteins involved in lipid binding. We established that there are two membrane proteins at 14 and 55 kDa that bind at least PE. We also showed that there are two lipid hydrolysing activities in the membrane by SDS-egg yolk overlay and by microtiter plate assays (PLC and lipase). Perhaps one or both proteins at 14 and 55 kDa may have PLC activity. Further experiments are needed to establish whether the 14 and 55 kDa proteins represent two different PLC's with different specificities, similar to *L. monocytogenes*, or whether one of the proteins has another function such as lipid transport. It would also be interesting to perform crosslinking experiments with an acylglycerol to determine the identity of the lipase. Once the enzymes are definitively identified, they will need to be purified and further characterized (lipid specificity, metal requirements, pH optimum, pI). Also, mutants deficient in each enzyme will need to be constructed in order to elucidate their roles (alteration of host cell membrane or mucus layer, cell signalling, escape from vacuole, nutrition). Further characterization of the potential lipase chaperone protein and lipid hydrolysing activities in the cell sonicates will also need to be done.

In chapter 5 we proposed that a *C. jejuni* 14 kDa protein may be involved in the induction of GBS. Further characterization of this 14 kDa protein is necessary in order to determine if this protein is the same protein identified in chapter 4 and whether it exhibits any similarities to the FABP family. It would be helpful to separate the *C. jejuni* membrane preparation by two-dimensional gel electrophoresis in order to determine the actual number of proteins in the 14 kDa region. Western blot analysis using antibodies against myelin P2 and the lipase chaperones could be used to specifically identify the 14 kDa proteins of interest. Also, reactivity with human antisera would demonstrate which protein(s) induces an immune response.

Whether the 14 kDa *C. jejuni* protein is involved in GBS remains to be determined. From the literature and from our results it appears that there is no single precipitating factor and that the development of GBS probably involves a combination of multiple antibody

insults, host immunity, and environmental factors. I only hope that researchers soon abandon the idea that gangliosides are involved in GBS and begin to look for other potential causes of GBS. In addition to determining how *C. jejuni* precipitates GBS, a more general question is how does *C. jejuni* cause diarrhea? Surprisingly, this question does not have an answer.

In the final chapter we investigated signal pathways that may be involved in attachment, invasion, and disease. Calcium appears to be important in *C. jejuni* infection but stronger data is required before any conclusions can be made. The same argument holds true for the involvement of microfilaments and microtubules in *C. jejuni* invasion. These findings should be confirmed by using different drugs with similar actions as well as using drugs with different mechanisms of action effecting the same signal.

As stated earlier, *Campylobacter* knowledge is still in its infancy. However, it is for this very reason that studying *Campylobacter* pathogenesis is so exciting. The progress in this area is rapidly advancing due to better genetic techniques and the renewed interest in studying *Campylobacter* due to its involvement in GBS. I predict that the next decade will unravel many of the mysteries we now have about how *Campylobacter jejuni* causes disease.